Translational research in ovarian carcinoma:

Cell biological aspects of drug resistance and tumor aggressiveness

Ate Gerard Jan van der Zee

RIJKSUNIVERSITEIT GRONINGEN

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Proefschrift

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Ate Gerard Jan van der Zee

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Co-promotor:	Dr. E.G.E. de Vries
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Aan het roer dien avond stond het hart
en scheepte maan en bossen bij zich in
en zeilend over spiegeling
van al wat het geleden had
voer het met wind en schemering
om boeg en tuig voorbij de laatste stad.

Gerrit Achterberg, 1955.
Voorbij de laatste stad.

Aan mijn ouders
Aan Joukje

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Contents

Voorwoord

Indien een proefschrift tot stand komt binnen een samenwerkingsverband van de Interne Oncologie, Gynaecologische Oncologie en Pathologie, betekent dit, dat een groot aantal mensen hieraan heeft bijgedragen. Het verrichten van onderzoek binnen een dergelijk samenwerkingsverband heeft, behoudens enkele kleine nadelen, vooral grote voordelen zoals de kennismaking met de verschillende denkculturen en specifieke vaardigheden binnen de diverse onderafdelingen en het vinden van vele mogelijkheden binnen en buiten het eigen ziekenhuis voor het realiseren van de diverse doelstellingen van het onderzoek.

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Introduction

Epithelial ovarian carcinoma has the highest mortality of all gynecologic malignancies in the Western world (1). The main reason for this important role of ovarian carcinoma in cancer deaths of women is the fact that 80-85% of women with ovarian carcinoma have advanced-stage disease at time of diagnosis due to the largely asymptomatic course of the disease. Prognosis for these patients is poor with an overall survival rate of 10-20% (2). In contrast, approximately 80-90% of patients with early-stage disease can be cured with (surgical) therapy (3). The most efficient way to improve the poor overall figures for patients with ovarian carcinoma seems to enlarge the low percentage of patients identified with early-stage disease by screening large cohorts of asymptomatic women. Until now no premalignant lesion of ovarian carcinoma has been identified. Therefore, the aim of screening should be the detection of the disease at an early stage. At present however, no effective tools are validated well enough to allow routine screening of (high risk) populations of women for the detection of ovarian carcinomas (4,5).

While in patients with early-stage disease surgical eradication is the mainstay of therapy, radical excision of all tumor is often impossible in patients with advanced-stage disease, because of metastases throughout the intra-abdominal cavity. To obtain cure in patients with advanced-stage disease chemotherapy has to be instituted after surgical debulking of the tumor. Unfortunately, current chemotherapy of epithelial ovarian carcinoma remains woefully inadequate. High response rates (65-80%) to platinum (Pt) based chemotherapy are found in primary patients, but the duration of response is too often brief. Response rates to chemotherapy in previously treated patients are low (0-40%), resulting in poor survival percentages (2,6,7).

These clinical data clearly show that in advanced-stage epithelial ovarian carcinoma intrinsic and acquired drug resistance are the main causes of ineffective therapy. Well-known prognostic factors with regard to survival in epithelial ovarian carcinoma are: stage, patient age, performance status, the amount of residual tumor after first laparotomy, absence or presence of ascites, tumor grade, histiotype, and DNA ploidy (8). Although these parameters do reflect biological features of both patients and tumors, they are not specifically related to the sensitivity or resistance of tumors to cytotoxic drugs. At present, the main guidelines for selecting drugs in the chemotherapeutic treatment of malignant (ovarian) tumors are clinical i.e. previous experience with drug treatment of the same type of tumors. Attempts have been made to develop in vitro chemosensitivity tests to individualize chemotherapeutic treatment and to avoid the use of drugs that will not have activity. Major theoretic and methodological objections to these methods exist such as selection of tumor cells, reproducibility, and tumor heterogeneity (9).

To improve the efficacy of chemotherapy in patients with epithelial ovarian carcinoma more understanding is needed of cell biological mechanisms involved in the presence or development of drug resistance. Optimal use of anti-cancer drugs requires both knowledge of classical pharmacological factors influencing drug efficacy, and knowledge of cell biological features related to drug sensitivity of the particular tumor being treated. Insight in these cell biological characteristics is rapidly emerging from in vitro studies, that use cultured tumor cells with intrinsic or acquired resistance to cytotoxic drugs as models. To translate this knowledge from in vitro studies to the complicated reality of tumor specimens and to evaluate their (prognostic) value in patients is an inevitable step in the search for the understanding and management of drug resistance in the clinic. The identification of cell biological parameters related to drug resistance in tumor specimens of patients will hopefully allow more rational design of chemotherapeutic treatment, and, if possible, individual tailoring of drug regimens, when clinical drug resistance occurs.

In this thesis diverse cell biological features that in cultured (ovarian) tumor cells have been linked to drug resistance and/or tumor aggressiveness are studied in tumor specimens of epithelial ovarian carcinomas.

Contents of the thesis

Multiple and sequential genetic changes occur in the course of (ovarian) carcinoma development. Malignant ovarian tumors comprise a reservoir of genetically heterogeneous tumor cells with varying capacities for growth, differentiation, metastasis and, most importantly, different sensitivities to cytotoxic drugs. In **chapter 1** genetic changes such as numerical alterations in chromosomes, overexpression of oncogenes, and loss of tumor suppressor genes in ovarian carcinoma are reviewed in possible relation to drug resistance.

Studies in cultured (ovarian) tumor cells have provided insight in the diverse mechanisms that may be involved in drug resistance. Cell biological factors involved in resistance to cytotoxic drugs can be found at different cellular levels: *1. the cell membrane:* membrane bound glycoproteins, such as P-glycoprotein (P-gp), are able to extrude natural drugs such as epipodophyllotoxins, vincristine, doxorubicin and taxol from the cell. Overexpression of P-gp in tumor cell membranes can confer the so-called multidrug resistance (MDR) phenotype; *2. the cytoplasm:* cytosolic non-proteins such as glutathione bind directly or by conjugation to drugs, such as Pt compounds and alkylating agents, and thereby prohibit the interaction of these drugs with their targets. Cytosolic enzymes such as glutathione S-transferases may catalyze the binding of drugs to glutathione. Enhanced expression of these cytosolic (non) proteins

mediates increased detoxification of various drugs; *3. the nucleus:* a: topoisomerase (Topo) I and II are nuclear enzymes, that are involved in DNA transactions such as transcription, replication, and mitosis. At the same time Topo I and II are the nuclear targets for cytotoxic drugs e.g. respectively, camptothecin and doxorubicin. In drug resistant tumor cells the levels of topoisomerases may be lowered (providing less target for the drug), or the enzymes may be altered by mutations in their genes, which make them insensitive for drugs; b: complex nuclear enzyme systems are involved in DNA synthesis and the repair of DNA damage. Resistance to DNA damaging drugs such as Pt compounds and alkylating agents can be conferred by the upregulation of these enzyme systems leading to an increased repair of DNA damage. In **chapter 2** these and other cell biological features that have been related to drug resistance in (ovarian) tumor cell lines are summarized and their appearance and potential relevance in human ovarian carcinoma are discussed.

In cultured tumor cells expression of P-gp and Topo I and II are important variables of resistance to natural cytotoxic drugs. In **chapter 3** the expression of P-gp and Topo I and II activity are evaluated in patients with benign ovarian tumors, primary ovarian carcinoma, and residual or relapsed ovarian carcinoma after Pt based chemotherapy. These data are correlated with histopathologic characteristics of the tumors such as differentiation grade, tumor volume index, and mitotic index of the tumors.

Apart from decreased Topo I and II levels (less target), qualitative alterations in Topo I and II can also lead to insensitivity of these drug targets. Two isozyme forms of Topo II have been described, Topo IIα and ß, that have different sensitivities to several antineoplastic drugs. Therefore, both the levels and ratio of Topo IIα and ß may be important factors in sensitivity of tumor cells to Topo II directed drugs. In **chapter 4** qualitative assays are used to investigate whether, in addition to quantitative differences, qualitative differences exist in Topo I and II in ovarian carcinomas before and after Pt based chemotherapy. Since the Topo II activity assay does not discriminate between Topo IIα or ß isozyme activity, immunoblotting with specific mono- and polyclonal antibodies is performed to detect these isozymes.

The Topo IIα gene and c-erbB-2 oncogene are both situated on chromosome 17q21-22. In breast adenocarcinomas it has been found recently that the Topo IIα gene can be coamplified with c-erbB-2 due to physical linkage of the two genes on chromosome 17q. As a consequence of coamplification, expression of the Topo IIα gene can be increased. Of particular relevance to Topo IIα expression is the reported amplification of c-erbB-2 in 20-30% of ovarian carcinomas. In addition, the long arm of chromosome 17 on which Topo IIα is located, frequently shows loss of heterozygosity in ovarian carcinomas and is the site of an as yet unidentified locus involved in familial breast and ovarian carcinomas. In **chapter 5** a detailed molecular analysis of the Topo IIα gene and its expression in ovarian carcinomas is carried out. Topo IIα expression is determined by immunoblotting. In addition, the Topo IIα locus on chro-

mosome 17 is analyzed in order to discover whether the expression of Topo IIα is influenced by gross genetic changes.

Glutathione S-transferases (GSTs) are a family of cytosolic proteins that are important enzymes of detoxification. In man, cytosolic GSTs have been divided into four major classes, termed alpha, mu, pi and theta. These isozymes are known to have different substrate specificities, and therefore both the total GST activity and the isozyme composition may be important determinants of a tumors' ability to detoxify different chemotherapeutic agents. In human tumor cell lines resistant to cisplatin and/or cyclophosphamide an enhanced GST content has been described. In **chapter 6** isozyme patterns of GSTs and their possible role in sensitivity of malignant ovarian tumors to chemotherapy are studied in benign ovarian tumors, untreated malignant tumors, and tumors after Pt containing chemotherapy.

Many studies on new prognostic factors in advanced-stage ovarian carcinoma are difficult to interpret because of unclear selection of patients, varying chemotherapy regimens, and lack of long term follow-up. In **chapter 7** the expression and prognostic value of P-gp, GST pi, the tumor suppressor gene p53 and the oncogene c-erbB-2 is retrospectively studied by immunostaining in a well documented, uniformly treated series of 89 patients with untreated advanced-stage ovarian carcinoma. The relations between the expression of P-gp, GST pi, p53, and c-erbB-2 and response to chemotherapy and survival are reported. Possible induction of expression of P-gp and GST pi is analyzed in residual tumors obtained at second look laparotomy after 3 cycles of chemotherapy.

High pretreatment platelet counts and low hemoglobin have recently been suggested as negative prognostic factors in patients with ovarian carcinoma (10). Interleukin-6 (IL-6) is a multifunctional cytokine with a diversity of functions leading to induction of C-reactive protein (CRP), increased platelet counts, and low hemoglobin. Different epithelial ovarian carcinoma cell lines produce varying amounts of IL-6. Elevated levels of IL-6 have been detected in ascitic fluid and serum from patients with ovarian carcinoma. In **chapter 8** a possible relation between IL-6 levels in cystic fluids of benign and malignant ovarian tumors and pretreatment serum CRP, platelet counts, and hemoglobin levels is evaluated.

Recently, Scheper et al described a monoclonal antibody against a M_r 110,000 vesicular protein, named *l*ung *r*esistance *p*rotein (LRP), which is overexpressed in P-gp negative multidrug resistant tumor cell lines (11). The LRP protein defines a drug resistance phenotype that is not mediated by P-gp. In **chapter 9** the expression and prognostic value of LRP is studied by immunohistochemistry in a series of 66 patients with advanced-stage ovarian carcinoma. Possible relations between the expression of LRP and response to chemotherapy and survival are described.

Finally, the results of our studies are summarized in **chapter 10**, and some perspectives for future studies are discussed.

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Chapter 1

Genetic changes in epithelial ovarian carcinoma in relation to drug resistance, a review

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Abstract

Objective *To review genetic changes in human ovarian carcinoma in relation to cytotoxic drug resistance.*

Methods *The occurrence of genetic changes in cultured (ovarian) tumor cells and tumor specimens from patients with ovarian carcinoma and their relation with the development of drug resistance are reviewed.*

Results *As spontaneous mutations in tumor cells occur approximately one in 10^4 - 10^6 cell divisions, the chances for a drug resistant mutation are directly related to the number of cells and thus the size of a tumor. Therefore, effort should be taken to remove as much tumor as possible at initial surgery for ovarian carcinoma. In human ovarian carcinoma high frequency of chromosomal loss has been observed at chromosome arms 6p, 17p, and 17q. No data exist on a possible relation of specific numerical chromosomal changes and clinical behavior or sensitivity to cytotoxic drugs. Overexpression of the c-erbB-2 oncogene and the epidermal growth factor receptor (EGF-R) has been reported in ovarian carcinomas in respectively 20-30% and 50-60%. Most studies show that c-erbB-2 and EGF-R overexpression may have a role in growth regulation in ovarian carcinoma. The administration of blocking monoclonal antibodies against these growth factor receptors in combination with cisplatin lead to additive cytotoxicity both in in vitro and in vivo studies. Upregulation of the c-fos, c-jun, and c-myc oncogenes, which are nuclear transcription factors, may occur after exposure of cultured tumor cells to cytotoxic drugs. Enhanced expression of these oncogenes may result in increased activity of DNA damage repair enzymes, that may be responsible for resistance to alkylating drugs such as platinum compounds. The role of upregulation of the c-fos, c-jun, and c-myc oncogenes in the clinical behavior of ovarian carcinoma, especially with regard to cytotoxic drug response, remains to*

be elucidated. Conflicting data exist on the role of the tumor suppressor gene p53 in the response of tumor cells to drugs. Theoretically loss of wild type p53 by mutation may result both in enhanced and decreased sensitivity to DNA damaging drugs, because of the role of p53 on the one side in cell cycle arrest and on the other side in apoptosis. Mutations in the p53 gene are found in 30-50% of human ovarian carcinomas. Positive p53 immunostaining, which is strongly related to the presence of p53 mutations is related to shorter survival, and more aggressive tumor growth, but not to poor response to chemotherapy.

Conclusions *The role of genetic changes in ovarian carcinoma with regard to tumor growth characteristics and response to chemotherapy is far from elucidated, but is gradually becoming more clear. The identification of a diversity of genetic changes and their consequences for clinical behavior such as response to cytotoxic drugs may offer opportunities for new approaches in the treatment of patients with ovarian carcinoma.*

Introduction

Overtime multiple genetic changes will occur during the development of human (ovarian) carcinomas. Genetic alterations may involve: 1. activation of oncogenes that determine transformation and invasiveness of cells; 2. inactivation of tumor suppressor genes that normally regulate or restrain cell division; and 3. inappropiate expression of growth factors (1).

Epithelial ovarian carcinomas are presumed to arise from the single layer of epithelial cells that cover the ovarian surface or ovarian inclusion cysts. Older epidemiologic data have linked malignant transformation of normal ovarian epithelial cells with "incessant ovulation". Long-time interruption of ovulation by use of oral contraceptives or by multiple pregnancies may protect women against developing ovarian carcinoma. Evidence for a link between "incessant ovulation" and malignant transformation also emerges from experimental studies by the occurrence of spontaneous malignant transformation in cultured normal rat ovarian epithelial cells (2-4).

Recent studies point to an unifocal origin of epithelial ovarian carcinomas (5,6). Additional mutations will occur in tumor development, resulting in a reservoir of genetically heterogeneous tumor cells with varying capacities for growth, differentiation, metastasis and sensitivity to cytotoxic drugs (7).

Patients with advanced-stage ovarian carcinoma can not be cured by surgery alone, because of early metastatic spread throughout the peritoneal cavity. Therefore, chemotherapy has a key role in the management of patients with advanced-stage ovarian carcinoma. Response rates to platinum-based chemotherapy as primary treatment vary from 60-80%, while response is significantly worse (0-40%) in patients with

residual or recurrent disease after previous chemotherapy. The poor outcome of patients with advanced-stage ovarian carcinoma is obviously due to intrinsic or acquired resistance to cytotoxic drugs (8).

The different degrees in resistance of ovarian carcinomas to cytotoxic drugs must be due to the occurrence of a variety of genetic changes. In this paper genetic changes in ovarian carcinoma will be reviewed with respect to their possible relation with drug resistance.

Genetic changes with regard to tumor volume

Goldie and Coldman showed that in murine tumors spontaneous mutations will occur approximately one in 10^4 - 10^6 cell divisions. Therefore, the chances for a drug resistant mutation are directly related to the size of a tumor (9). No randomized prospective studies have been performed, which address the issue of a beneficial effect of extensive removal of tumor load at first operation in patients with advanced-stage ovarian carcinoma (10). Recently a prospective randomized study reported a beneficial effect of intervention surgery after three cycles of chemotherapy in patients with advanced-stage ovarian carcinoma with regard to progression free survival (11). The results of this study confirm the theoretical considerations of the advantages of cytoreductive surgery such as removal of poorly perfused, bulky tumor masses, the existence of higher growth fractions in the small, better perfused, residual tumor masses, and removal of drug resistant tumor cells (12). Based on these theoretical considerations and clinical evidence it must be the first aim to obtain an optimal tumor debulking at first laparotomy for ovarian carcinoma.

Numerical genetic changes

A recent review on genetic changes in epithelial solid neoplasia showed that in epithelial tumors gene deletion, implying loss of negative regulation of proliferation, is the predominant mechanism in their origin as well as progression (13). Numerical genetic changes in human malignancies can be detected by cytogenetic and loss of heterozygosity studies. Cytogenetic studies can detect gross losses, large deletions and/or translocations of chromosomes. Loss of heterozygosity studies using polymorphic genetic markers can detect the same lesions as well as lesions in smaller chromosome regions that may harbor e.g. tumor suppressor genes of interest. It has been shown that the extent of underlying chromosomal damage is related to stage and degree of malignancy. Lowry et al found loss of a greater part of chromosome 17 in high grade and advanced-stage ovarian tumors in comparison to low grade tumors

(14,15). As most cytogenetic and loss of heterozygosity studies have been performed on small series of specimens from advanced-stage and high grade ovarian tumors a segregation of changes potentially related to progression of disease is not possible (13,16).

Table 1 Numerical genetic changes in ovarian carcinoma and relation to clinicopathologic prognostic factors.

Cytogenetic studies	
frequent deletions in chromosome arm	
1p, 3p, 6q, 11p, 19p [13,16]	
LOH# studies[17-19]	
"high" frequency* of LOH# at chromosome arms	
in all three studies	
6p, 17p, 17q	
in two studies	
7p[17,18], 15q & 22q[18,19]	
in one study	
8q, 12[17]	
5q, 6q, 8p, 9q, 14q, 18q, 21q[18]	
13q[19]	
"High" frequency* of LOH# of chromosome arm	**Associated with**
13q, 15q[19]	high grade tumors
3p[19]	low grade tumors
11p[21]	high grade tumors
6q, 17q[20,21]	serous tumors

x: reference number; #: LOH: loss of heterozygosity; *: definitions of "high" were different in all studies.

Numerical genetic changes in ovarian carcinoma, as reported in cytogenetic studies and in loss of heterozygosity studies in three larger series of patients are summarized in table 1 (13, 16-21). Different frequencies of loss of heterozygosity of specific chromosome regions in these studies may be due to the use of different genetic markers, different composition of the patient cohorts with regard to tumor grade and stage, and different definitions of high frequency of loss of heterozygosity. Only at chromosome arms 6p, 17p, and 17q high frequency of loss of heterozygosity was found in all three studies.

For relations between numerical genetic changes and histiotype or tumor grade, see table 1. A relation of specific patterns of numerical genetic changes and sensitivity

to cytotoxic drugs has not been reported. Studies in large series of patients, who are well documented with regard to clinicopathologic prognostic factors are necessary to identify numerical genetic changes in ovarian carcinomas which may have clinical relevance.

Changes in oncogenes

The number of identified oncogenes is still growing rapidly and has passed over 100 (1). In the following paragraphs only those oncogenes will be discussed, which have been studied in human ovarian carcinoma and have been related to drug resistance.

Genes encoding for tyrosine kinases

The c-erbB-2 oncogene (also known as HER2/neu oncogene), located on chromosome 17q21, encodes for a transmembrane phosphoglycoprotein with intrinsic tyrosine kinase activity. It has close structural similarity to the epidermal growth factor receptor (EGF-R)(22). Ligands have been identified that specifically bind to the c-erbB-2 receptor, and these will either promote differentiation or stimulate cell growth (23,24). Amplification or overexpression of the c-erbB-2 oncogene may lead to increased tyrosine kinase activity and, as a consequence, more aggressive tumor growth (25). In cultured tumor cells from diffrent histogenetic origin c-erbB-2 overexpression was found to decrease the sensitivity of tumor cells to cytotoxic drugs such as cisplatin, doxorubicin, and melphalan (26). In a recent clinical study it was shown that breast carcinomas with c-erbB-2 overexpression were less responsive to chemotherapy (27). How c-erbB-2 overexpression can confer chemoresistance is unclear.

In table 2 the frequency of c-erbB-2 amplification and/or overexpression in human ovarian carcinomas and its possible prognostic relevance with regard to survival are summarized for studies comprising more than 50 patients. A wide range exists both in the reported frequency of c-erbB-2 amplification and/or overexpression in ovarian carcinomas and in their prognostic significance (28-44). These conflicting data may be due to the use of different methodologies for the determination of c-erbB-2 amplification and/or overexpression, subjectivity of scoring, patient selection, and different treatment regimens (45). Recently, we have found c-erbB-2 overexpression in 20% of 89 patients with advanced-stage ovarian carcinoma. In this study c-erbB-2 overexpression was neither related with response to platinum containing chemotherapy nor with prognosis as expressed by progression free and overall survival (43).

From most recent studies it appears that, independent from treatment given, c-erbB-2 overexpression per se is not a prognostic factor in ovarian carcinomas. However, at the same time most studies show that in an important percentage of ovarian carcinomas c-erbB-2 overexpression may have a role in growth regulation.

Therefore, the findings of Hancock et al who showed in ovarian carcinoma cell lines that blocking of c-erbB-2 overexpression with anti-c-erbB-2 antibodies resulted in enhanced cytotoxicity of cisplatin, may have clinical importance in the future (46).

Table 2 C-erbB-2 amplification and/ or overexpression and relation to clinicoprognostic factors and survival in human ovarian carcinomas.

Ref.no.	n	material	method	% pos.	related to
27	87	frozen	SB	26	survival ↓[1]
42	86	frozen	SB	1	-
27	67	frozen	NB	34	survival ↓[1]
37	105	frozen	IHC	24	-
27	72	frozen	IHC	50	survival ↓[1]
33	72	frozen	IHC	43	-
35	243	paraffin	IHC	19	survival ↓[1]
38	94	paraffin	IHC	35	-
43	89	paraffin	IHC	20	undifferentiated histiotype
39	74	paraffin	IHC	5	-
41	56	paraffin	IHC	18	-

Ref.no.: reference number; n: number of tumors; SB: Southern blotting; NB: Northern blotting; IHC: immunohistochemistry; % pos: percentage of positive tumors; CT: chemotherapy; 1: in univariate analysis; -: no relation found; ↑: longer survival; ↓: shorter survival.

The EGF-R is a membrane associated glycoprotein, consisting of an external ligand binding domain, a single transmembrane section and an internal domain which displays specific tyrosine kinase activity (47). Binding of EGF or transforming growth factor alpha (TGFα) to the EGF-R induces a variety of intracellular changes i.e. stimulation of DNA synthesis and cell proliferation (48). In two human ovarian carcinoma cell lines EGF-R activation was found to be associated with enhanced sensitivity to cisplatin. Which EGF-induced change was responsible for the sensitization to cisplatin remained unclear (49). In human ovarian carcinomas the frequency of EGF-R positive tumors ranges from 31-77% (50-56)(see table 3).

Bauknecht et al initially reported a higher response rate to platinum containing chemotherapy and longer overall survival in patients with EGF-R positive ovarian carcinoma (50). In a later study no prognostic benefit with regard to survival was found anymore in the EGF-R positive group (51). Recently, the same author reported higher response rates to platinum containing chemotherapy for EGF-R positive tumors (52).

Table 3 EGF-R expression and relation to clinicoprognostic factors and survival in human ovarian carcinomas.

Ref.no.	n	material	method	% pos.	related to
51	111	frozen	LBA	33	response to CT ↑ survival ↓[2]
57	105	frozen	LBA	49	ND
56	100	frozen	LBA	66	ND
50	84	frozen	LBA	36	response to CT ↑ survival ↑[1]
54	55	frozen	LBA	31	NR
57	105	frozen	IHC	41	ND
56	100	frozen	IHC	77	ND
55	92	frozen	IHC	NS	survival ↓[2]
53	87	frozen	IHC	77	survival ↓[1]
54	35	frozen	IHC	57	response CT ↓

Ref.no.: reference number; n: number of tumors; NR: no relation found; LBA: ligand binding assay; IHC: immunohistochemistry; % pos: percentage of positive tumors; CT: chemotherapy; [1]: in univariate analysis; [2]: in multivariate analysis; ↑: better (response to chemotherapy or longer (survival); ↓: worse (response to chemotherapy) or shorter (survival); ND: no correlation study performed; NS: not stated.

In contrast, several other groups found a worse prognosis for patients with EGF-R positive tumors (53-55). Discrepancies in the reported frequencies of EGF-R positive tumors and its prognostic value may be due to the different techniques used to determine EGF-R expression i.e. ligand binding assay versus immunohistochemistry, patient selection and different treatment regimens. Studies in which both methodologies were compared showed concordance of 70 to 21% for both assays (56,57). Foekens et al found no difference in prognosis for patients with EGF-R positive and negative ovarian carcinoma as determined by ligand binding assay, while patients with EGF-R positive ovarian carcinoma as determined by immunohistochemistry had a worse prognosis (54). Only in one study the prognostic value of EGF-R expression was determined by multivariate analysis. In this study positive EGF-R expression was found to be an independent negative prognostic factor (55).

In vitro and in vivo studies have shown that anti-EGF-R monoclonal antibodies block the binding of EGF or TGFα to their receptors, resulting in growth inhibition, but not in cell death (58,59). Combined treatment of anti-EGF-R monoclonal antibodies with cisplatin or doxorubicin resulted in stronger antitumor activity than with chemotherapy or anti-EGF-R monoclonal antibodies as single agents (60,61). It is currently unknown which mechanism is reponsible for this additive cytotoxic effect.

More insight is needed in the mechanisms of the putative relation of EGF-R activation and response to chemotherapy. Also more clinical studies, using both metho-

dologies for measurement of EGF-R expression are needed to evaluate the prognostic impact of EGF-R expression in ovarian carcinoma. However, combination of anti-EGF-R monoclonal antibodies plus chemotherapy may be a novel form of therapy for the many human ovarian carcinomas which express the EGF-R.

Genes encoding for G proteins

Active ras oncogenes can modulate cell growth by regulating signal transduction at the cell membrane. Mutations in one of the three ras genes, Ha-ras, Ki-ras, and N-ras, or enhanced expression of the normal p21-ras products through gene amplification or erroneous transcription, may convert these genes into "active" oncogenes (62-64). Studies in murine fibroblasts have linked transformation by mutant c-Ha-ras oncogenes with increased resistance to cisplatin, accompanied by decreased cellular drug accumulation and enhanced detoxification by increased cytosolic metallothionein content (65,66). In contrast, Perez et al observed no influence on cisplatin sensitivity by transfer of mutant ras genes in cultured rat epithelial ovarian and fibroblast cells (67).

In earlier studies ras gene amplification or mutation appeared to be uncommon in human ovarian carcinoma with a combined 5-10% incidence (68). In recent studies a higher incidence of Ki-ras mutations (26-42%) has been reported (69,70). Ki-ras mutations are found in mucinous ovarian carcinomas (46-75%), which represent 15% of all ovarian carcinomas (69,71). Conflicting data in the literature on the frequency of Ki-ras mutations in ovarian carcinomas are probably due to varying numbers of tumors with mucinous histiotype in the different studies. Scambia et al found higher ras protein (p21) expression in malignant ovarian tumors in comparison to benign tumors, using an antibody which recognizes all ras proteins (normal and mutant) by immunoblotting and immunohistochemistry. P21 overexpression was not related to response to chemotherapy, and had a minor negative impact on overall survival (72). Different results have been reported by Rodenburg et al, who found no prognostic value for p21 overexpression by immunohistochemistry (73).

At present there seems to be little evidence that activation of ras genes either by mutation or amplification can contribute to cisplatin resistance (67). In ovarian carcinomas the clinical significance of amplified or mutated ras genes is still unclear, but seems to be limited to tumors with a mucinous histiotype. It remains to be established whether the worse prognosis for patients with mucinous ovarian carcinoma is linked to the frequent appearance of ras mutations.

Genes coding for nuclear transcription factors

The jun, fos and myc oncoproteins act as nuclear transcription factors that regulate the expression of a variety of genes important in normal cellular growth and differentiation processes. C-jun, c-fos and c-myc genes can be activated by the exposure of cells

to a variety of extracellular stimuli, such as growth factors, and chemotherapeutic agents (74,75). Scanlon et al provided evidence that in human ovarian carcinoma cell lines fos expression may (in part) be responsible for directing the cellular response to DNA damage by cytotoxic agents. Upregulation of fos expression after exposure of tumor cells to cisplatin results in enhanced levels of DNA damage repair enzymes, such as thymidylate synthase, DNA polymerase ß, and topoisomerase I and enhanced levels of metallothioneins, which are small cytosolic proteins involved in cellular heavy metal detoxification. Overexpression of these enzymes/proteins may result in drug resistance by enhanced repair of DNA damage or increased detoxification (76-78). Sklar et al demonstrated in erythroleukemia cell lines a direct correlation of c-myc expression and cisplatin resisance. Their findings suggested a possible role for c-myc in the regulation of specific DNA repair pathways (79). Exposure of human myeloid leukemia cells to cisplatin resulted in increased expression of the c-jun gene, that was accompanied by the occurrence of internucleosomal DNA cleavage characteristic of programmed cell death (80).

Data on c-fos, c-jun, and c-myc in human ovarian carcinoma are rather scarce. Bauknecht et al reported very infrequent (< 8%) DNA amplification of c-jun and c-myc (52). Collected data from earlier reports in small series of patients reported a higher (33%) frequency of c-myc amplification (68). At the protein level Bauknecht et al found overexpression of c-myc and c-jun in 28% and 38% of untreated tumors, respectively. One study in a larger series of patients showed c-myc overexpression in 37% of primary ovarian carcinomas, while the prognostic impact of c-myc overexpression was not evaluated (81). Scanlon et al reported higher c-fos expression in cisplatin-resistant tumors after platinum chemotherapy in comparison to untreated tumors (77).

Bauknecht et al separated 32 ovarian carcinomas in tumors that have low and high expression of c-jun and c-myc. Tumors with high expression of these nuclear transcription factors were found to respond better to platinum based chemotherapy (52). Except for the study by Bauknecht no other studies exist on the prognostic impact of c-fos, c-jun, and c-myc expression in ovarian carcinomas. The role of these nuclear transcription factors in the clinical course of ovarian carcinoma has to be explored in larger series of patients to obtain conclusive information on their perhaps important role in response to chemotherapy.

Changes in tumor suppressor genes

Retinoblastoma (RB) gene

The RB gene is a tumor suppressor gene located on chromosome 13q14 (82). Loss of RB expression has been linked to increased tumor aggressiveness in bladder carci-

noma (83). Independent studies recently reported normal RB protein expression in the majority (96%) of ovarian carcinomas (84-86). Despite the fact that abnormalities of chromosome 13 (see paragraph on cytogenetics) are frequently found in ovarian carcinomas the RB gene itself does not seem to be critically involved.

p53

The product of the p53 tumor suppressor gene is a nuclear phosphoprotein that is involved in cell cycle control. Normally, wild type p53 exerts a growth-inhibitory activity. Loss of wild type p53 function by different pathways (such as mutations) may lead to more aggressive tumor growth (87). In addition to its established role in growth-regulation recent studies have shown that levels of wild type p53 increase rapidly after radiation- or drug-induced DNA damage. Increased wild type p53 levels will result in cell cycle arrest, and thereby facilitate DNA damage repair (88,89). In vitro studies showed that cells with loss of wild type p53 function were more sensitive to DNA-damaging agents (90,91). Based on these observations, the presence of p53 mutations, causing loss of wild type p53 function, may result in an increased sensitivity to chemotherapy. Brown et al have observed that transfection of mutant p53 into ovarian carcinoma cell lines resistant to cisplatin indeed conferred an increased sensitivity to cisplatin. However, transfection of this mutant p53 in sensitive ovarian carcinoma cell lines did not affect sensitivity at all (89). In contrast, loss of wild type p53 function has been reported to increase resistance to a variety of cytotoxic drugs (92). This increased resistance has been linked to the loss of the presumed triggering role of wild type p53 in the process of apoptosis. Slichenmyer et al failed to identify activation of p53-dependent cell death pathways after exposure to cytotoxic drugs, but found that DNA damage elicited wild type p53-dependent cell cycle arrest (93,94). However, by transfering wild type p53 into a p53 defective (by allelic loss) human non-small cell cancer cell line Fujiwara et al showed an increase in sensitivity to cisplatin which was found to be related to the promotion of apoptosis (95). It remains to be elucidated if sensitivity to cytotoxic drugs will change when normal p53 is re-introduced in tumor cells that have loss of p53 function by mutations in the p53 gene.

Changes in the p53 tumor suppressor gene are among the most common genetic alterations found in human malignancies (96). We found p53 immunostaining in 20% of stage I/II and 40% of stage III/IV ovarian carcinomas (43), which is comparable to results of other studies, in which the frequency of p53 immunostaining ranged from 15-29% in stage I/II and 40-62% in stage III/IV ovarian carcinomas (97-105). Positive immunostaining of p53 in ovarian carcinomas has been found to be strongly associated with the occurrence of missense mutations (101). In most human malignancies such as carcinoma of the breast, lung, colon, and prostate, positive p53 immunostaining (as an indicator of mutation) is a negative prognostic factor (106-110). In our study patients with p53 positive ovarian carcinoma had significantly shorter progres-

sion free and overall survival. Positive p53 immunostaining was associated with poorly differentiated tumors, the presence of ascites, and large residual tumor after first laparotomy, and not with response to chemotherapy. The negative prognostic impact of positive p53 immunostaining in ovarian carcinoma appears therefore to be due to more aggressive tumor growth, and not to resistance to cytotoxic drugs. In a multivariate analysis positive p53 immunostaining did not retain its independent prognostic significance. Our results are in contrast with the first report on p53 immunostaining in ovarian carcinoma patients in which no prognostic significance of p53 immunostaining was found (98). In this study tumor specimens used for p53 determination were not always obtained before exposure to chemotherapy, which may have influenced p53 expression. Recently, two other studies also reported a negative prognostic role for positive p53 immunostaining in ovarian carcinomas (104,105).

The presumed negative prognostic significance of positive p53 immunostaining in different malignancies, and the possible role of p53 in response to chemotherapy have lead to the design of in vitro studies in which the defective gene is replaced by the insertion of the normal tumor suppressor gene. The major problem of this kind of gene therapy in solid tumors lies in the delivery of actively expressed vectors to every tumor cell in vivo (111). However, this approach may especially be feasible in ovarian carcinomas, for which the negative prognostic value of positive p53 immunostaining has been confirmed, and in which the spread of the tumor is largely limited to the intraperitoneal cavity, which is easily accessible for "drug" administration.

Changes in peptide growth factors and cytokines

Peptide growth factors and cytokines may have a growth stimulating or inhibiting effect on tumor cells. Aberrant expression of peptide growth factors may activate autocrine or paracrine loops, resulting in growth advantage of malignant cells compared to benign cells (112). A variety of peptide growth factors, cytokines and their receptors have been identified in ovarian carcinomas, such as TGF ß, EGF, platelet-derived growth factor (PDGF), macrophage/monocyte colony-stimulating factor, interleukin (IL) -1, IL-6, IL-10, and tumor necrosis factor α (113-115). Overexpression of several growth factors and/or their receptors such as EGF-R and PDGF-Rα, has been related to shorter survival in ovarian carcinomas. Only EGF-R (over) expression has been linked to sensitivity to platinum compounds in ovarian carcinoma patients. Bauknecht has found that an activated EGF/EGF-R complex, leading to higher fos and myc expression, was related to higher sensitivity to chemotherapy (52). These results are contradictory to the hypothesis of the group of Scanlon et al (52),who found higher fos expression to be related to resistance to platinum com-

pounder and also to the observation that blocking the EGF-R by monoclonal antibodies enhances the cytotoxicity of cisplatin.

Table 4 Relations of genetic changes and resistance to cytotoxic drugs.

Genetic change	cell lines	human tumor specimens
numerical	ND	ND
oncogenes		
c-erbB-2 amplification	+[26,27]	+/-[28-44]
EGF-R overexpression	+[49]	+/-[50-54]
Ras mutations	+/-[65-67]	-[72,73]
Fos overexpression	+[78]	+/-[76,77]
Jun overexpression	+[80]	-[52]
Myc overexpression	+/-[79]	-[81]
tumor suppressor genes		
Rb	ND	-[85,86]
p53	+/-[88-95]	-[44,104,105]

ND: not done; -: existing data do not point to a relation; +/-: varying results in different studies; +: existing data point to a relation; [x]: reference number.

Conclusions

Data on genetic changes in ovarian carcinoma is growing exponentially. It is largely unknown whether any of these changes are involved in tumorigenesis or are mainly associated with tumor progression. In table 4 the putative relations between genetic changes and resistance to cytotoxic drugs are summarized. From experimental studies it appears that aberrant expression of diverse oncogenes and/or tumor suppressor genes in (ovarian) tumor cells may lead to altered interactions with cytotoxic drugs. However, these data still are scarce, and far from equivocal. Most studies have focussed on the relation of changes in one oncogene or tumor suppressor gene with drug resistance. In malignant tumor cells more genetic changes accumulate in the course of the disease. Therefore, studies which focus on multiple genetic changes in tumor cells and drug resistance are warranted.

Limited data exist in ovarian carcinoma specimens to confirm the prevalence and relevance of these genetic changes in the clinic. Evaluation of changes in the respective oncogenes and tumor suppressor genes in larger series of well documented and uniformly treated patients with advanced-stage ovarian carcinoma are needed. Especially, attention has to be paid to the variety of methodologies that are used to determine the respective parameters, and consensus should be reached on which methodologies should preferably be used.

Despite this lack of information, it still emerges from this review that the reported changes in oncogenes and tumor suppressor genes in ovarian carcinomas provide opportunities to develop novel mechanisms to treat malignant tumors. The sensitivity of malignant tumors to cytotoxic drugs may be enhanced by combining cytotoxic drugs with monoclonal antibodies against overexpressed oncogenes or growth factor receptors such as c-erbB-2 and EGF-R. The fact that e.g. the c-erbB-2 oncogene is overexpressed in malignant tumors and not in normal tissues, may enlarge the therapeutic window of these cytotoxic drugs, when they are combined with monoclonal antibodies against c-erbB-2.

Loss of wild type p53 function by mutations in the p53 tumor suppressor gene is a frequent event in ovarian carcinomas, and appears to have a negative impact on prognosis with regard to survival. Reintroduction by gene therapy of the normal p53 protein into tumor cells with mutant p53 may have a growth inhibitory effect. At the same time also the sensitivity to drugs may be altered (111). The relation of p53 function and drug resistance is far from elucidated and more conclusive data are needed with regard to this issue.

More insight in the genetic changes that result in the different phenotypes of malignant (ovarian) with regard to tumor aggressiveness and resistance to cytotoxic drugs hopefully will lead to therapies, that are targeted to parameters uniquely expressed in malignant cells, and therefore will be more effective than the currently used agents, that lack this specificity.

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Chapter 2

Cell biological markers of drug resistance in ovarian carcinoma, a review

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Abstract

Objective *To review the mechanisms of resistance to the four classes of drugs that are widely used in ovarian carcinoma: platinum (cisplatin/carboplatin) compounds, classical alkylating agents (cyclophosphamide/melphalan), natural drugs (doxorubicin), and "new drugs" (taxol and its synthetic analogue taxotere).*

Methods *The literature comprising studies in cultured (ovarian) tumor cells and studies in tumor specimens from patients with ovarian carcinoma is reviewed with regard to identified mechanisms of drug resistance.*

Results *Both platinum and classical alkylating agents mediate their cytotoxicity by the formation of drug-DNA-adducts, resulting in DNA damage. Therefore, drug resistance mechanisms are (in part) comparable. In ovarian carcinoma cell lines increased repair of DNA damage and increased detoxification by binding of drugs to glutathione, possibly catalyzed by glutathione S-transferases have been identified as the most prominent resistance mechanisms to these drugs. Studies on the role of DNA repair mechanisms and glutathione in human ovarian carcinoma are hampered by the complexity of enzyme systems involved in DNA repair and intratumor heterogeneity for glutathione. Resistance to doxorubicin appears to be mediated by enhanced efflux from the cell by increased expression of membrane glycoproteins acting as a drug efflux pump, such as P-glycoprotein. Resistance to doxorubicin can also be due to quantitative and/or qualitative changes in the nuclear target of doxorubicin, topoisomerase (Topo) II. Finally, resistance to taxol may be mediated by enhanced expression of P-glycoprotein, while presumed other mechanisms such as alterations in tubulin structure, the cellular "target" of taxol, and changes in polymerization of tubulin are still largely unresolved. Several ways to modulate the reviewed resistance mechanisms are also described.*

Conclusions *This review shows that many cell biological factors may be involved in*

drug resistance. The relevance of the identification of most of these factors in ovarian carcinoma patients remains to be established.

Introduction

Meta-analyses of randomized clinical trials have shown, that platinum compounds, alkylating agents and anthracyclines are the most active drugs in ovarian carcinoma (1-3). A recent meta-analysis by Levin et al showed a dose-intensity versus response relation for platinum compounds used as single agents or in combination with other drugs. A similar relation was found for cyclophosphamide used as a single agent, and for doxorubicin used in combination with platinum compounds (4). An upper limit for the response to carboplatin in advanced ovarian carcinoma has been suggested (5). Therefore, it was considered that further gains in chemotherapy may be achieved by escalating the dose of cyclophosphamide, or, preferably, the dose of doxorubicin within multi-agent platinum-based regimens (4). These considerations were confirmed in a recent consensus meeting on the treatment of advanced ovarian carcinoma (6).

Another strategy for improvement of the efficacy of chemotherapy in ovarian carcinoma is to include "new" drugs with unique mechanisms of action such as taxol or its semi-synthetic analogue taxotere (7). Phase II trials on efficacy of taxol as monotherapy in patients with relapsed or refractory ovarian carcinoma have documented response rates of 20-35% (8). In a recent phase III trial first line treatment of taxol in combination with cisplatin was compared with a combination of cisplatin and cyclophosphamide. The overall response rate of the taxol-cisplatin group was higher than that of the cisplatin-cyclophosphamide group and the median period of progression free survival was significantly longer in the first group. Data on survival are expected at the end of 1994 (8).

Despite improvements in median progression free survival as result of the above mentioned strategies, it is obvious that intrinsic and/or acquired resistance to well known drugs such as platinum compounds, alkylating agents, anthracyclines and "new" drugs such as taxol and taxotere will remain the major obstacles in the management of patients with ovarian carcinoma. Both intrinsic and acquired resistance to cytotoxic drugs result in the poor overall survival figures for these patients (5-year survival: 10-20%)(10).

In cultured (ovarian) tumor cells various cell biological mechanisms have been identified that may have a role in drug resistance as encountered in the clinic. In this paper the mechanisms of resistance to four classes of drugs are reviewed, that are widely used in ovarian carcinoma patients. Their prevalence and possible clinical relevance in ovarian carcinoma patients are also discussed. Finally, possible ways to modulate these mechanisms of resistance are mentioned.

Resistance to platinum compounds

Recent studies suggest that the cytotoxicity of platinum compounds is mediated by the induction of apoptosis, as a result of lethal DNA damage caused by platinum-DNA-adducts (11). Cytotoxicity of platinum compounds is probably a function of drug influx, drug efflux, the extent of platinum-DNA-adduct formation, and the rate at which DNA damage will be repaired. Various reviews have summarized the factors involved in platinum resistance (12-15). For a summary, see table 1. At present the dominant mechanism responsible for clinically acquired resistance is unclear. Possible mechanisms of resistance to platinum compounds as identified in (ovarian) tumor cell lines can be divided in decreased net drug accumulation, altered drug inactivation, and increased repair of DNA damage (12).

Table 1 Mechanisms of resistance to platinum compounds and their possible relevance in human ovarian carcinoma cell lines and tumor specimens

Mechanism	evidence in cell lines/ xenografts	evidence in human tumors
Decreased accumulation	+	ND
Increased detoxification		
glutathione mediated	++	+/-
GST mediated	+/-	+/-
MT mediated	-	-
Increased DNA repair	+	+/-

-: no evidence; +/-: only circumstantial evidence such as higher expression levels of the parameter in resistant cell lines or tumors is available and varying results in different studies; + : most studies point to an important role; ++: evidence obtained by transfection studies or specific depletion studies; ND: no data available.

Decreased platinum compound accumulation.

Much controversy in the literature exists on the way platinum compounds enter the tumor cell. Recently, it was postulated that approximately one half of the initial drug uptake rate will be due to passive diffusion, and that the other half is occurring by facilitated diffusion through a gated channel (16). In human ovarian carcinoma cell lines decreased cellular accumulation of platinum compounds has been found to be in part responsible for the resistant phenotype (17-19). Due to practical limitations in the determination of platinum levels in malignant tumors the importance of decreased accumulation as mechanism of drug resistance in the clinic still remains to be established.

Studies of (non-ovarian) cisplatin-resistant cells with decreased drug accumula-

tion have identified two different cell-membrane proteins that may be involved in platinum compounds uptake and efflux, respectively (20,21). No data exist on the prevalence of these two membrane proteins in ovarian carcinoma cell lines or tumor specimens.

As more information evolves on the mechanisms of platinum transport more rational ways can be found to modulate this transport. In vitro data show a variety of compounds, that more or less effectively can modulate platinum cytotoxicity, all interfering at different levels of platinum transport (16,22). However, until more information becomes available on the importance of decreased platinum accumulation in the clinic it does not seem rational to enter these platinum transport modulating agents in clinical trials.

Enhanced platinum compound inactivation

Glutathione is a tripeptide thiol, which has an important role in the cellular detoxification of various xenobiotics, such as platinum compounds (23). How glutathione can influence platinum-induced cytotoxicity and confers resistance to platinum compounds is not completely clear. Recently, Ishikawa provided evidence for direct binding of cisplatin to glutathione in a glutathione-cisplatin chelate complex. Elimination of this complex from tumor cells by a glutathione S-conjugate export pump reduces the intracellular accumulation of platinum compounds (24,25). In a preliminary study it was shown that glutathione and this export pump both are overexpressed in cisplatin-resistant human leukemia cells (26). In this model glutathione protects the cell by intercepting reactive platinum complexes before they can react with DNA. With regard to the generality of this model one may object that decreased accumulation of platinum compounds is not frequently observed in platinum resistant ovarian carcinoma cell lines in contrast to enhanced glutathione content (12).

Glutathione synthesis requires the formation of precursors such as glutamate by the cell membrane-bound enzyme gamma-glutamyl transpeptidase (GGT) (27). Successive actions of the cytosolic enzymes gamma-glutamylcysteine synthetase (GCS) and glutathione synthetase subsequentely catalyze the synthesis of glutathione. The first glutathione synthesis step, catalyzed by GCS, is rate-limiting and can be inhibited by buthionine sulfoximine (28). In human ovarian carcinoma cell lines a strong relation has been found between levels of glutathione and glutathione synthesis enzymes (GCS, GGT) with sensitivity to platinum compounds. At the same time resistance to platinum compounds can be reversed by depleting high glutathione levels in ovarian carcinoma cells with buthionine sulfoximine (29,30). However, several platinum resistant human ovarian carcinoma cell lines have been described that do not show an elevated glutathione level (12). It still may be that glutathione elevation in platinum resistant cell lines turns out to be a non-specific response to any selection pressure (31). Experiments in which cisplatin-sensitive cells with low glutathione levels are

transfected with cDNA of the glutathione synthesis enzyme GCS may shed more light on this issue. In this way the effects of increased cellular glutathione levels on drug resistance can be studied in the absence of other potential contributors to resistance.

Only limited data exist on glutathione levels in human ovarian carcinoma samples. The assessment of glutathione levels in tumor biopsies is associated with technical problems (32) and is also hampered by significant intratumor heterogeneity in glutathione levels (33,34). In unpaired samples Britten et al found higher glutathione levels in specimens from patients after chemotherapy in comparison to untreated patients (35). Hanigan et al determined GGT expression in benign and malignant human ovarian tumors. The GGT level may be a marker of the cellular capacity for glutathione synthesis, which may be an even more important parameter than steady-state glutathione levels. Data on a possible relation of GGT expression and response to chemotherapy are not yet available (36).

Apart from a direct binding of platinum compounds to glutathione, binding of platinum compounds to glutathione may also happen by conjugation, mediated by glutathione S-transferases (GSTs). GSTs are cytosolic proteins that function as enzymes of detoxification by catalyzing the conjugation of electrophilic agents such as platinum compounds to glutathione (37). In man, cytosolic GSTs have been divided into four major classes termed alpha, mu, pi, and theta (38,39). As these isozymes are known to have different substrate specificities, both total GST activity and GST isozyme composition may be determinants of a tumor's ability to detoxify chemotherapeutic agents (40). In several platinum resistant human tumor cell lines an enhanced GST content has been described (41). However, in two different panels of ovarian carcinoma cell lines no relation was found between GST levels and resistance to platinum compounds (29,30). Gene transfection studies did not show a clear role for GSTs in platinum resistance (for summary of GST transfection studies, see ref. 42).

Data on GSTs in human ovarian carcinoma are also conflicting. Using high pressure liquid chromatography we found GST pi to be the predominant GST isozyme in ovarian carcinomas (43), which has also been reported by others (44,45). GST pi levels in untreated tumors were not related to response to chemotherapy. Lower or comparable GST pi levels have been found in tumors after chemotherapy in comparison to untreated tumors (43,45). In a retrospective study in another series of patients with advanced ovarian carcinoma we found no relation between GST pi immunostaining and response to chemotherapy or survival (46). Our findings are in contrast with a study by Green et al, who found high intensity of GST pi staining to be related to resistance to cytotoxic chemotherapy and shorter overall survival (47). Overall it appears from in vitro studies and (most) data in human ovarian carcinoma that GST levels are not an important marker of platinum resistance in human ovarian carcinoma.

Metallothioneins are small cytosolic proteins involved in cellular heavy metal detoxification (48). Platinum compounds have been shown to bind to metallothioneins (49). The evidence on the role of metallothioneins in platinum resistance is weak. In most platinum resistant ovarian carcinoma cell lines metallothioneins levels are unchanged (12). Transfection experiments with a metallothionein gene construct yielded inconsistent effects on cisplatin resistance (50,51). In malignant ovarian tumors Murphy et al found neither changes in metallothioneins levels induced by chemotherapy, nor a relation between metallothioneins content and response to chemotherapy. However, the number of patients in this study was small and no sequential biopsies were studied (52).

Enhanced repair of DNA damage, caused by platinum compounds

Repair of DNA damage caused by platinum-DNA-adducts occurs primarily by the nucleotide excision repair pathway (53). It is estimated that a large number of enzymes are involved in this pathway (54). Several platinum-resistant ovarian carcinoma cell lines show increased DNA repair, as determined by enhanced loss of platinum adducts and increased synthesis of DNA repair enzymes (12,55). A variety of individual DNA repair enzymes have been linked to platinum resistance in ovarian and other tumor cell lines (56-58). Scanlon et al showed that exposure of tumor cells to platinum compounds results in upregulation of the c-fos oncogene. Increased expression of this nuclear transcription factor results in higher levels of enzymes involved in DNA repair such as thymidylate synthase, DNA polymerase ß, and topoisomerase I (59).

Besides increased DNA repair in the total genomic DNA, preferential repair of transcriptionally active genes has also been reported (60). In a series of cisplatin-resistant ovarian carcinoma cell lines Johnson et al found increased removal of platinum compounds from total genomic DNA to be associated with increased resistance. Repair of interstrand cross-links in the resistant cell lines was also more apparent in actively transcribed sequences (55).

Enhanced levels of glutathione have also been reported to be involved in increased DNA repair (61). Possible mechanisms are: glutathione prohibits the formation of platinum-DNA- intra- or interstrand cross-links after the formation of platinum-DNA-monoadducts; glutathione stabilizes DNA repair enzymes such as DNA polymerase α; and finally, glutathione facilitates the synthesis of DNA precursors such as deoxyribonucleotide triphosphates, that are essential for DNA repair (62,63).

The complexity of the DNA repair mechanism offers major problems to evaluate DNA repair capacity in tumor specimens. In human ovarian carcinoma the human DNA repair gene ERCC-1 was found to be expressed at higher levels in clinically drug resistant tumors than in responsive tumors. However, the number of patients was small, and patient selection was not defined (58). Bramson et al recently demonstrated

that transfection of the ERCC-1 gene in Chinese hamster ovary cells lead to overexpression of ERCC-1 and, surprisingly, increased sensitivity to cisplatin and melphalan. These findings illustrate the complex nature of DNA repair mechanisms (64). It also implicates that it is questionable to link the expression of one or two of the many enzymes involved in DNA repair to clinically encountered resistance to cytotoxic drugs.

An advantage of the fact that many proteins are involved in DNA repair is, that this offers many opportunities to modulate resistance to platinum compounds. Different drugs such as aphidicolin, interfere at different levels in DNA repair and have been found to modulate platinum compounds cytotoxicity in tumor cell lines (19). Phase I clinical trials of aphidicolin showed that prolonged steady-state levels can be achieved at concentrations proven to be maximally inhibiting the repair of platinum-induced DNA damage (65). Recently, O'Dwyer et al found in mice superior activity of the combination of aphidicolin and platinum compounds in comparison to platinum compounds alone against cisplatin-resistant human ovarian carcinoma xenografts (66).

Apart from proteins more directly involved in DNA repair, a fast growing number of nuclear proteins are identified that bind to platinum-damaged DNA. The function of these proteins is still largely undefined and may vary from shielding platinum-DNA adducts from repair to signalling cells into apoptosis after the recognition of DNA damage (12). Bissett et al demonstrated the presence of cisplatin-DNA-damage recognition proteins in ovarian carcinoma cell lines and in extracts prepared from biopsies of ovarian malignancies. Neither in cell lines nor in tumor extracts a relation was found between the amount of damage recognition proteins and sensitivity to cisplatin (67).

Enhanced repair of DNA damage caused by platinum compounds is very frequently encountered in cultured platinum-resistant ovarian carcinoma cells, and also appears to occur early in the acquisition of resistance of tumor cells to platinum compounds. Therefore, this mechanism of resistance appears to be most important. However, its previously mentioned complex nature prohibits the evaluation in the clinic.

Platinum-DNA-adducts determination after in vivo exposure to platinum compounds

The assumption that platinum-adduct formation in non-tumor cells and tumor cells is comparable has resulted in patient studies in which platinum-DNA-adduct formation in easily obtainable non-tumor cells was related to response of the tumor to chemotherapy. This putative relation of platinum-DNA-adduct formation in non-tumor cells with response to chemotherapy implies that response to platinum-based chemotherapy is a function of the patient's innate capacity to repair DNA, which may be deter-

mined by inheritance or by exposure to environmental factors prior to treatment (68).

Studies in patients with ovarian carcinoma, and with a variety of other types of malignancies have confirmed that levels of platinum-DNA damage determined in non-tumor cells (leukocytes, buccal cells) are highest in patients whose tumors respond to platinum chemotherapy (68-73). In responders platinum-DNA-adduct formation is presumed to be higher and/or platinum-DNA-adduct retainment to be longer. From two studies it appears that the relationship between platinum-DNA-adduct levels and response is lost with subsequent drug administrations (68,71). Moreover, important overlap in initial platinum-DNA-adduct levels in responders and non-responders raises doubts on the use of platinum-DNA-adducts measurements in non-tumor cells to predict subsequent response to chemotherapy in individual patients. The development of methods to visualize and monitor in situ-induced platinum-DNA-adducts in easily accessable malignant tumors such as cervical carcinomas may prove to be more effective (71,74).

Resistance to alkylating agents

Classical alkylating agents such as cyclophosphamide and melphalan, which are particularly used in ovarian carcinomas, form monofunctional or bifunctional bonds with DNA (75). The resultant interstrand DNA cross-links appear to be the major lesions mediating cytotoxicity (76). As both classical alkylating agents and platinum compounds mediate their cytotoxicity by the formation of drug-DNA-adducts, the drug resistance mechanisms are (in part) comparable (see table 2 for summary). In platinum resistant ovarian carcinoma cell lines cross-resistance to alkylating agents such as melphalan is frequently found (30,55,77). Cellular resistance to alkylating agents has generally been attributed to mechanisms which either detoxify the agent or repair DNA damage. However, also changes in drug transport have been described (78).

Decreased alkylating agents accumulation

Alkylating agents may enter cells by a variety of mechanisms. Melphalan uptake has been attributed to two aminoacid transport systems (79). Recently, Moscow et al described the first in vitro model of transport-associated melphalan resistance in a human (breast carcinoma) cell line (80). In human ovarian carcinoma cell lines or tumor specimens differences in accumulation of alkylating agents have not been described (81), and therefore the role of this mechanism in human ovarian carcinoma is undefined.

Table 2 Mechanisms of resistance to alkylating agents and their possible relevance in human ovarian carcinoma cell lines and tumor specimens.

Mechanism	evidence in cell lines/ xenografts	evidence in human tumors
Decreased accumulation	-	ND
Increased detoxification		
glutathione mediated	++	++
GST mediated	+/-	+/-
ALDH mediated	ND	+/-
Increased DNA repair		
ATase	ND[1]	+/-
excision repair	ND	-

-: no evidence; +/-: only circumstantial evidence such as higher expression levels of the parameter in resistant cell lines or tumors is available and varying results in different studies; + : most studies point to an important role; ++: evidence obtained by transfection studies or specific depletion studies; ND: no data available; [1]: transfection studies in cell lines originating from other malignancies confer resistance.

Enhanced alkylating agent inactivation

Glutathione appears to be important in the detoxification of alkylating agents. Conjugates of melphalan and cyclophosphamide with glutathione have been demonstrated (for summary, see ref. 82). In different human ovarian carcinoma cell lines resistance levels to alkylating agents were related to intracellular levels of glutathione, and glutathione synthesis related enzymes. Depletion of glutathione levels by buthionine sulfoximine significantly reversed resistance to these drugs (29,30,77,81,83). Studies in tumor-bearing nude mice also showed enhanced cytotoxicity of melphalan in combination with buthionine sulfoximine (84,85). These observations have resulted in the design of phase I clinical studies of melphalan in combination with buthionine sulfoximine. Substantial depletion of cellular glutathione levels (as determined in peripheral mononuclear cells) could be obtained in patients (86-88). Interestingly, it was observed in mice that maintaining buthionine sulfoximine in the drinking water gave the largest therapeutic benefit when combined with melphalan (85).

GST levels were not found to be increased in different platinum resistant ovarian carcinoma cell lines cross-resistant to melphalan (29,30,77,81,83). In transfection studies the most convincing evidence for a role in alkylating drug resistance has been found for the GST alpha isozyme (for summary, see ref. 42,89). In human ovarian carcinoma specimens we found that GST pi and not GST alpha is the predominant GST isozyme, before and after cyclophosphamide containing chemotherapy (43). Some in vitro studies showed that ethacrynic acid, as inhibitor of GST, can modulate resistance to

alkylating agents (19). These observations have lead to phase I clinical trials of ethacrynic acid in combination with thiotepa (90). The lack of convincing evidence for an important role of GSTs in drug resistance to alkylating agents in human ovarian carcinoma makes the use of modulating agents such as ethacrynic acid not very promising in these patients.

Detoxification of cyclophosphamide can also be mediated by aldehyde dehydrogenase (ALDH). This cytosolic enzyme catalyzes the conversion of aldophosphamide (activated cyclophosphamide) to (inactive) carboxyphosphamide (91). Elevated levels of ALDH correlated with resistance to cyclophosphamide in different (non-ovarian) tumor cell lines (82,92,93). In a small series of fresh biopsies of untreated human ovarian tumors Djuric et al found low ALDH levels, while ALDH levels were slightly increased in (unpaired) tumors after chemotherapy (94). No studies on ALDH levels are performed in larger series of patients.

Enhanced repair of DNA damage, caused by alkylating agents

Repair of DNA damage, caused by alkylating agents occurs both by DNA base repair and nucleotide excision repair. DNA base repair enzymes such as O^6-alkylguanine-DNA-alkyltransferase (ATase) can remove alkyl groups from damaged DNA. In different (non-ovarian) tumor cell lines resistant to alkylating agents such as nitrosurea, ATase has been found to be overexpressed (95,96). Transfection of ATase cDNA in ATase deficient cells conferred resistance to alkylating agents (97). No data exist on ATase in human ovarian carcinoma cell lines. Lee et al reported on high ATase expression in human ovarian carcinoma in comparison to Hodgkin's lymphoma. It was suggested that the reported low efficacy of nitrosurea in ovarian carcinoma in comparison to high efficacy in Hodgkin's lymfoma may be due to different ATase levels in these tumors (98)

Nucleotide excision repair pathways are also involved in repair of DNA damage by alkylating agents; a variety of enzymes have been found to be involved (53,64,99). The importance of these enzymes in conferring clinical drug resistance to alkylating agents remains to be established, and will be hard to elucidate, because of the aforementioned complex nature of DNA repair.

Resistance to doxorubicin

Mechanisms of cellular resistance to doxorubicin can be categorized as follows: 1: decreased drug accumulation by enhanced efflux of drugs, mediated by P-glycoprotein or other drug transporters; 2: increased detoxifying capacity by upregulation of glutathione and/or GST levels; 3: decreased DNA damage by quantitative and/or

qualitative changes in the nuclear target of doxorubicin: topoisomerase (Topo) II (see table 3 for summary).

Table 3 Mechanisms of resistance to doxorubicin and their possible relevance in human ovarian carcinoma cell lines and tumor specimens

Mechanism	evidence in cell lines/ xenografts	evidence in human tumors
Decreased accumulation		
P-glycoprotein mediated	+	+/-
MRP	ND[1]	ND
LRP	ND	ND
Increased detoxification		
glutathione mediated	+	ND
GST mediated	+/-	-
Changes in Topo II	ND	+/-[2]

-: no evidence; +/-: only circumstantial evidence such as higher expression levels of the parameter in resistant cell lines or tumors is available and varying results in different studies; + : most studies point to an important role, and/or expression levels in primary tumors are related to response to chemotherapy; ++: evidence obtained by transfection studies or specific depletion studies; ND: no data available; [1]: transfection studies in cell lines originating from other malignancies confer resistance; [2]: data are available in ovarian carcinoma that show varying levels of Topo II and no indications for qualitative changes in Topo II. No data exist on Topo II and response to doxorubicin in ovarian carcinoma.

Decreased accumulation of doxorubicin

P-glycoprotein is a membrane glycoprotein acting as an efflux pump for certain classes of unrelated drugs, such as doxorubicin and epipodophyllotoxins i.e. natural products. The overexpression of P-glycoprotein in tumor cell lines selected for resistance to a single drug is accompanied by cross-resistance to other natural products, resulting in the so-called multidrug resistance (MDR) phenotype (100). Transfection of the MDR-1 cDNA into drug-sensitive cells confers the MDR phenotype (101). In several human ovarian carcinoma cell lines resistant to doxorubicin, overexpression of P-glycoprotein has been found to be (in part) responsible for reduced cytotoxicity of doxorubicin (102-105). Data on the frequency of P-glycoprotein expression in human ovarian carcinoma and its possible prognostic significance are conflicting. In an immunohistochemical study we found positive P-glycoprotein immunostaining in 15% of untreated ovarian carcinomas (46). Other studies reported lower (6 and 7%) expression rates of P-glycoprotein in ovarian carcinomas using immunohistochemistry and Northern blotting, respectively (106,107). Higher incidences of P-glycoprotein expression (80 and 73%) were found in studies using the polymerase chain reaction, which is a very sensitive detection technique (108,109). Arao et al compared

P-glycoprotein expression as determined by polymerase chain reaction and conventional immunohistochemistry in ovarian carcinomas. Only the tumor with the highest P-glycoprotein expression as determined by polymerase chain reaction showed positive immunostaining (110). The wide range in reported frequencies of P-glycoprotein expression in ovarian carcinomas is obviously due to the sensitivities of the detection techniques used.

In some human malignancies elevated expression of P-glycoprotein has been linked to unresponsiveness to (MDR related) chemotherapy and dismal prognosis (111). In an uniformly treated (cisplatin, cyclophosphamide, doxorubicin) series of 89 patients with advanced ovarian carcinoma we found positive P-glycoprotein immunostaining neither to be related with response to chemotherapy, nor with survival (46). In a smaller study Holzmayer et al found high expression (determined by polymerase chain reaction) of P-glycoprotein to be related to failure to response to chemotherapy, which was even more apparent in patients treated with MDR related drugs (109). Arao et al did not find a relation between P-glycoprotein expression levels and response to chemotherapy (110). The problem of both studies is that the clinical information is incomplete and patient selection undefined, which weakens the strength of the observations.

We found higher frequency (48%) of P-glycoprotein immunostaining in tumor specimens after doxorubicin containing chemotherapy in comparison to specimens from untreated patients (15%), which is in agreement with other studies (107,112). Induction of P-glycoprotein immunostaining by doxorubicin containing chemotherapy points to a role of P-glycoprotein in acquired drug resistance in ovarian carcinoma.

In phase I and II trials in a variety of malignancies attempts have been made to modulate clinical MDR by co-administration of non-cytotoxic inhibitors of P-glycoprotein, e.g. verapamil (113,114). In ovarian carcinoma patients Ozols et al were unable to demonstrate potentiation of the activity of doxorubicin by verapamil in drug resistant tumors, which were not evaluated for P-glycoprotein expression (115). As the role of P-glycoprotein in drug resistance in ovarian carcinoma is not clearly defined it seems sensible to limit new modulator studies to malignancies in which (classic) natural drugs have a more prominent role in chemotherapeutic regimens than in ovarian carcinomas (117).

Recently, a new membrane associated drug transporter protein, *MDR*- associated *P*rotein (MRP), has been identified, which is associated with resistance to doxorubicin and other natural drugs, and is overexpressed in non-P-glycoprotein expressing tumor cell lines with MDR phenotype (117,118). Gene transfection studies showed that MRP, like P-glycoprotein can actually confer resistance to a wide spectrum of natural drugs (119,120). No data exist on the prevalence of MRP in ovarian carcinoma cell lines or tumor samples.

Scheper et al have developed a monoclonal antibody against a vesicular protein, named *l*ung *r*esistance *p*rotein (LRP), which is (like MRP) overexpressed in P-glycoprotein negative tumor cell lines but with the MDR phenotype (121). In a preliminary study in untreated advanced-stage ovarian carcinomas we found positive LRP immunostaining to be strongly related to response to (mainly) platinum-based chemotherapy. Moreover positive LRP immunostaining was found to be the strongest prognostic factor with regard to survival in uni- and multivariate analysis (122). More information on the function of LRP should elucidate the background of the prognostic impact of LRP in ovarian carcinomas, while on the other hand the preliminary results of our retrospective study have to be confirmed in a prospective study in a larger series of patients.

Enhanced doxorubicin inactivation

Cytotoxicity of doxorubicin appears to be in part linked to the generation of free oxygen radicals, which lead to cell injury and death. A major mode of action of glutathione is the scavenging of toxic free radicals in the environment of critical target sides such as DNA (41). In human ovarian carcinoma cell lines enhanced steady-state levels and synthesis capacity of glutathione have been linked to resistance to doxorubicin (77,123).

Maeda et al found enhanced GST pi expression in a doxorubicin resistant human ovarian carcinoma cell line, which resistance could be partially reversed by W-77, a GST activity inhibitor (107). However, reversal of resistance was also due to the fact that W-77 is extruded from the cell by P-glycoprotein, which was overexpressed in the resistant cell line. Transfection studies of GST pi cDNA into breast carcinoma cell lines showed that GST pi overexpression alone does not confer resistance to doxorubicin (124). These in vitro data present an uncertain role for GST isozymes in resistance of ovarian carcinoma cells to doxorubicin, which has been confirmed in our study in human ovarian carcinoma specimens (46).

Changes in the target of doxorubicin: Topo II

Topo II is a nuclear enzyme involved in various DNA transactions such as replication, transcription, and recombination. Topo II targeted drugs such as doxorubicin produce stabilized doxorubicin-Topo II-DNA cleavable complexes, that result in cell death (125). Apart from decreased Topo II levels, providing less target for the drug, qualitative alterations in Topo II may result in insensitivity of the target for the drug. For the Topo II enzyme two isozymes, Topo IIα and ß, have been described, that have different affinities to Topo II targeted drugs. Changes in the ratio of Topo IIα and ß may also be responsible for altered sensitivity of tumor cells to Topo II targeted drugs (126,127). No data exist on Topo II levels in doxorubicin resistant human ovarian carcinoma cell lines. Hamaguchi et al showed that cross-resistance to doxorubicin in cis-

platin resistant ovarian carcinoma cell lines was not due to alterations in Topo II expression (77).

We found higher Topo II activity in malignant human ovarian tumors in comparison to benign tumors, and 16- fold differences in Topo II activity in the malignant tumors. Tumors after platinum-based chemotherapy had lower Topo II activity than untreated tumors (128). In subsequent studies we showed that Topo IIα protein levels are not regulated by DNA amplification and that Topo II isolated from human ovarian carcinomas is able to form cleavable complexes in the presence of Topo II targeted drugs. The determination of cleavable complex formation, as mediator of cytotoxicity, in tumor extracts seems at present the most reliable parameter of Topo II expression in human malignancies to be related to response to Topo II targeted chemotherapy. Different levels of Topo II in ovarian carcinomas may (in part) be responsible for the variable response of ovarian carcinomas to Topo II targeted drugs (128-130).

Resistance to taxol

Taxol (paclitaxel) and its synthetic analogue taxotere (docetaxel) represent a novel class of drugs acting by promotion of the polymerization of microtubules and by inhibition of their depolymerization. In vitro the degree of microtubule bundling after taxol exposure has been found to be correlated with cytotoxicity. As for almost all drugs, cytotoxicity of taxol was found to be in part mediated by signalling cells into apoptosis (131-133). Until now three mechanisms of resistance to taxol have been described: P-glycoprotein mediated increased efflux, alterations in tubulin structure, and changes in levels of tubulins or polymerization of tubulins (see table 4).

Altered transport of taxol

In a variety of tumor cell lines resistance to taxol has been linked to an increased expression of P-glycoprotein. Reversal of this resistance could be obtained using modulators of P-glycoprotein (134-137). In fresh ovarian carcinoma specimens Eck et al found, that P-glycoprotein expression in the tumor cells was associated with response to taxol (138). In human ovarian carcinoma P-glycoprotein is not frequently expressed in primary tumors (see paragraph on the accumulation of doxorubicin). However, treatment with taxol may induce P-glycoprotein expression, as has been reported for other natural products (46,109,114). Consequently, efforts to modulate P-glycoprotein mediated resistance to taxol may become more clinically relevant.

Alterations in tubulin structure

Cabral et al described altered α and ß tubulin in mutant Chinese hamster ovary cell

Table 4 Mechanisms of resistance to taxol and their possible relevance in human ovarian carcinoma cell lines and tumor specimens

Mechanism	evidence in cell lines/ xenografts	evidence in human tumors
Decreased accumulation P-glycoprotein mediated	+[1]	+[1]
Alterations in tubulin structure	ND	ND
Changes in tubulin levels or polymerization of tubulins	+[1]	+/-[1]

-: no evidence; +/-: only circumstantial evidence such as higher expression levels of the parameter in resistant cell lines or tumors is available and varying results in different studies; + : most studies point to an important role; ++: evidence obtained by transfection studies or specific depletion studies; ND: no data available; [1]: only preliminary data exist.

lines after they had acquired resistance to taxol (139). Surprisingly, these resistant cell lines require the presence of taxol to grow, which has also been described for other taxol resistant cell lines (140). Whether this phenomenon also occurs in human ovarian carcinoma cell lines or tumors is unknown.

Changes in tubulin levels or polymerization of tubulins

Zahn et al showed in several taxol resistant cell lines decreased tubulin levels. After taxol treatment a shift to polymerized tubulins was observed in sensitive, but not in taxol resistant cell lines. In human breast and ovarian carcinomas a broad range of tubulin expression was found (141). These results suggest that resistance to taxol may be mediated by alterations in tubulin levels or adaptations in the dynamics of tubulin polymerization.

Discussion and conclusions

This review shows that many mechanisms at different cellular levels may be involved in resistance to cytotoxic drugs. Changes in several parameters are present at the same time in highly resistant tumor cell lines. This observation has resulted in the hypothesis that drug resistance is multifactorial. However, the majority of the reviewed parameters have been discovered and studied in cultured tumor cells selected for their high levels of resistance. Resistance levels in the clinic are probably much lower, which implies that in patients perhaps only single mechanisms will be responsible for resistance (31). Since increased repair of DNA damage caused by platinum com-

pounds or other alkylating agents occurs early and consistently during selection of resistant cell lines this mechanism of resistance appears to be activated first (12). The complexity of DNA repair mechanisms hampers the evaluation of this mechanism in the clinic. However, DNA repair inhibiting agents that are targeted to rate-limiting enzymes of DNA repair may show efficacy in the clinic in the future.

The majority of evidence for a possible role of the respective cell biological parameters in drug resistance is based on changes in these parameters in resistant cells in comparison to responsive cells. However, these changes may very well be due to coamplification or coactivation of the parameter studied together with other unknown, but more relevant factors. This implies that only induction of resistance by transfection of cDNA of the involved parameter into sensitive tumor cells can prove its role in drug resistance (31). In fact, this kind of evidence has sofar only been presented for P-glycoprotein and MRP in resistance to doxorubicin, and for ATase in resistance to nitrosurea.

Increased cytosolic glutathione levels have been linked to resistance to platinum compounds, alkylating agents, and doxorubicin. Hamaguchi et al showed that in ovarian carcinoma cell lines primary resistant to cisplatin, the cross-resistance to drugs such as melphalan and doxorubicin is only correlated with enhanced intracellular glutathione levels (77). This phenomenon reflects the encountered broad cross-resistance in patients with treatment refractory ovarian carcinoma after platinum chemotherapy. Despite the support for an important role for glutathione in resistance to a variety of cytotoxic drugs, transfection experiments with the recently obtained cDNA encoding for glutathione synthesis enzymes still have to confirm this. However, most studies indicate that modulation of glutathione metabolism may be one of the most promising means of reversing clinical drug resistance. In ongoing phase I clinical trials melphalan is administered in combination with buthionine sulfoximine. Results with regard to efficacy of this combination in phase II trials have to be awaited.

Resistance as a result of decreased intracellular levels of drugs mediated by unknown cell membrane proteins (platinum compounds, alkylating agents) or well characterized cell membrane glycoproteins such as P-glycoprotein (doxorubicin, taxol) are frequently encountered in cultured tumor cell lines. The role in the clinic of these cell membrane (glyco)proteins remains to be established. Especially in solid tumors one may presume that physiological barriers e.g. in poorly-vascularized regions of the tumor are also critical in obtaining cytotoxic intracellular drug levels (144).

This review shows that the understanding of cell biological mechanisms of drug resistance is developing fast. However, the relevance of this knowledge for the management of patients with drug resistant ovarian carcinoma remains to be established. Studies in larger, unselected, uniformly treated, and well documented series of patients are needed to evaluate the clinical relevance of parameters of drug resis-

tance. Consensus should be reached on which methodologies should be used to determine the expression of the respective parameters. The use of different methodologies is probably one of the main reasons for conflicting data on the clinical relevance of drug resistance parameters e.g. P-glycoprotein.

The different phenotypes of ovarian carcinoma patients with regard to sensitivity to chemotherapy must be due to the variety of genetic changes that occur during the development of ovarian carcinomas. In chapter 2 of this thesis the possible relation of genetic changes in ovarian carcinoma and drug resistance has been reviewed. It appears that aberrant expression of oncogenes and/or tumor suppressor genes may not only lead to changed growth characteristics, but also to altered interactions with cytotoxic drugs. However, data with regard to the link between genetic changes and mechanisms of drug resistance still is scarce, mainly limited to platinum compounds, and far from equivocal. Further elucidation of the possible links between frequently observed genetic changes in ovarian carcinoma such as c-erbB-2 amplification or p53 mutations and parameters of drug resistance is important because it may offer ways to manipulate drug resistant tumors.

It is hoped that the translation of insights in mechanisms of drug resistance from in vitro studies into the clinic will facilitate more effective chemotherapy. Identification of specific parameters of drug resistance or the genetic changes that are responsible for them, may lead to a more rational, perhaps individualized choice of chemotherapy regimens or to the administration of specific resistance modulating agents.

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Chapter 3

P-glycoprotein expression and DNA topoisomerase I and II activity in benign tumors of the ovary and in malignant tumors of the ovary, before and after platinum/cyclophosphamide chemotherapy

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Abstract

P-glycoprotein (P-gp) expression and DNA topoisomerase (Topo) II are important variables in multidrug resistant tumor cell lines. The aim of this study was to evaluate P-gp expression and Topo I and II activity in benign and malignant epithelial ovarian tumors. P-gp expression was analyzed immunohistochemically in cryostat sections of fresh tumor specimens. In the same specimens Topo I and II activity were measured by respectively relaxation of supercoiled plasmid BR322 DNA and decatenation of kinetoplast DNA. P-gp expression (range: 5-100% positive staining cells) was found in 3 of 6 cystadenomas, 0 of 2 borderline tumors, 15 of 21 untreated ovarian carcinomas, and 8 of 13 platinum/cyclophosphamide treated ovarian carcinomas. Median Topo I and II activity were elevated in malignant ovarian tumors compared to benign and borderline tumors. No difference was found between median Topo I activity in untreated ovarian carcinoma and Pt / Cy treated ovarian carcinoma. High Topo II activity ($\geq 8 \times 10^2$ U/mg protein) was more frequent in untreated compared to platinum/cyclophosphamide treated samples. Respectively, 8- and 16-fold differences in Topo I and II activity were found in the malignant tumors. Topo II activity in malignant tumors correlated with Topo I activity ($r=0.36$, $p<0.05$), and the tumor volume index ($r=0.35$, $p<0.05$). However this last weak correlation can not explain the 16-fold differences in Topo II activity in malignant tumors. Mitotic index, and P-gp expression did not correlate with Topo I or II activity. A large variability in P-gp expression and Topo I and II activity was observed in patients with ovarian carcinoma.

Introduction

Ovarian carcinoma is the leading cause of gynaecologic cancer death in the Western world (1). Chemotherapy is the treatment of choice in these patients when surgical resection is not considered to be curative. Nowadays, first line chemotherapy in ovarian carcinoma consists of a platinum (Pt) analogue in combination with cyclophosphamide (Cy) with response rates of 48-76% (2). Other chemotherapeutic agents such as anthracyclines and epipodophyllotoxins have been shown to be active against ovarian carcinoma with response rates of 23 to 36% (3). However, the majority of patients will die of their disease (4). These clinical data suggest that an intrinsic and acquired resistance to chemotherapy must occur in ovarian carcinoma. Our aim was to study the expression of P-glycoprotein (P-gp), and DNA topoisomerase (Topo) I and II activity in benign and malignant epithelial ovarian tumors in order to evaluate possible variables related to the resistance of ovarian carcinoma to drugs such as anthracyclines and epipodophyllotoxins.

In several human tumor cell lines selection for resistance to a single natural product drug resulted in cross-resistance to other natural products, the so-called multidrug resistance (MDR), which is associated with the increased expression of cell membrane P-gp, resulting in an increased efflux of the natural drugs. Resistance to drugs involved in MDR such as anthracyclines and epipodophyllotoxins can also be associated with alterations in the target of these drugs, Topo II, which mechanism has been called atypical MDR (5,6). Knowledge of these variables, P-gp and Topo II, in human tumors is increasing, but still limited. In recent studies, expression of P-gp in tumor cells, measured with immunohistochemistry appeared to be an important adverse prognostic factor concerning response to natural products in different human malignancies (7-9). With respect to ovarian carcinoma previous studies, using Northern and slot blot hybridization techniques showed no P-gp mRNA expression in untreated ovarian carcinoma (10,11). Immunological detection of P-gp has been performed in three small series of ovarian carcinoma (12-14), and in one larger series (15). These studies showed conflicting data, concerning the expression of P-gp in ovarian carcinoma. Chan et al showed in human ovarian cell lines that immunocytochemical detection of P-gp, using a three step immunoperoxidase technique was as sensitive as Northern blot and more sensitive than standard Western blot (16). Therefore we chose to study the expression of P-gp in ovarian tumors using the same immunohistochemical technique.

For the study of atypical MDR Topo II was determined, and because of close relation with Topo II also Topo I. Topos I and II are nuclear enzymes involved in the regulation of DNA topology, and have been identified as the targets of several anticancer drugs (17). Topo I for camptothecin and camptothecin derivates, Topo II for anthracyclines, epipodophyllotoxins, acridines, mitoxantrone and ellipticine (18-21). Resis-

tance to topoisomerase inhibitors has been attributed to quantitative and/or qualitative changes in both topoisomerases in several cell lines (22-26). Thus far, hardly any data exist on Topo I and II activity in solid human tumors, and no data are available on Topo I and II activity in ovarian carcinoma.

All the patients, from whom tumor samples were taken after chemotherapy, received Pt/Cy containing chemotherapy. Until now no data are available to show a relation between P-gp expression and resistance to platinum or alkylating agents. However, in cell lines conflicting data exist on Topo II in resistance to platinum and alkylating agents. Topo II might have a role in DNA repair by making DNA damage accessible to repair enzymes (27). In cell lines with an acquired resistance to Pt increased and decreased Topo II activity have been described (28,29). In cell lines with an acquired resistance to alkylating agents increased Topo II activity has also been reported (27,30). Therefore, we determined Topo I and Topo II activity in residual or relapsed ovarian carcinoma after Pt/Cy chemotherapy. All these data were correlated with tumor volume index, and mitotic index of the tumors.

Materials and methods

Monoclonal antibody, DNA, cell lines, and chemicals

The monoclonal antibody used for P-gp detection was C219 (Centocor Diagnostics, Malvern, PA). Form I kinetoplast DNA (kDNA) was isolated from the mitochondria of *Crithidia fasciculata* and purified by CsCl/ethidium bromide centrifugation as described previously (25). The supercoiled dimer of plasmid BR322 DNA was prepared from *Escherichia coli* strain HB 101. Plasmid BR322 DNA was isolated according to the alkaline lysis method and purified by CsCl/ethidium bromide centrifugation as described before (25). Myeloma cell lines with absent (8226S), low (8226Dox 4-6), and high (8226 Dox 40) P-gp expression were kindly provided by Dr. W. Dalton (31). Phenylmethanesulfonyl fluoride (PMSF) was obtained from Merck, Darmstadt, Germany.

Human material

Tumor specimens were obtained from tumors operated at cooperating hospitals in the northern part of the Netherlands during the period 1987-1990. The tumor collection was supervised by a pathologist. The samples for immunohistochemistry and topoisomerase extraction were immediately frozen in liquid nitrogen, and stored at -180°C until further analysis. In two patients untreated ovarian carcinoma tumor specimens were obtained from two different sites of the same tumor. In one patient untreated ovarian carcinoma tumor specimens were obtained from the ovarian tumor and a metastasis in the omentum at the same time. In one patient tumor specimens were obtained before and after chemotherapy.

Pathological characteristics

The tumors were histologically classified according to the World Health Organisation classification using paraffin embedded tissue sections (32). One section per cm tumor diameter was made to get a good overall impression of the tumor histology. Differentiation grade, tumor volume index, and mitotic activity index were measured in the paraffin embedded sections. The tumor volume index (percentage of malignant epithelial tissue in tumor specimen), was measured by a point counting technique, using a 42-point grid placed on a projection microscope x 200 as described by Baak et al(33). The mitotic index was calculated by counting the number of mitotic figures in ten high power fields at x 400.

Immunohistochemistry

Cryostat sections were used for immunohistochemistry. Cryostat sections of the tumor specimens were made (6 μm), allowed to dry for 1 h, then fixed in cold acetone (-20°C) and stored at -80°C until examination. For P-gp detection the C219 monoclonal antibody was used as described by Kartner et al(34). Immunostaining was performed with a three step immunoperoxidase technique using rabbit anti-mouse antibody and swine anti-rabbit peroxidase conjugated antibody. The peroxidase staining was performed with 3-amino-9-carbazole and H_2O_2 for 15 min. Sections were counterstained with hematoxylin. Negative controls were performed, using an irrelevant monoclonal antibody. All sections were read independently by two pathologists (HH and AG). The percentage of positive staining tumor cells was estimated. The intensity of the staining was scored semi-quantitatively in three classes (-, +, and ++) by matching the intensity of the staining of the tumor cells to the intensity of staining of two myeloma cell lines with a well established, increasing P-gp expression, namely + for 8226 Dox 4-6, and ++ for 8226 DOX 40. The parental, drug-sensitive cell line 8226S was used as negative control (31). Whenever the observers differed in the percentage of C219 positive tumor cells or intensity of staining the mean percentage and intensity were recorded for statistical analysis. Although in all sections important regional heterogeneity in the presence and intensity of P-gp expression was detected, an overall percentage positive cells was estimated, and the highest intensity of staining seen, was scored by the observers. Staining patterns were discriminated in cytoplasmatic (c), membrane bound (m), or both (c/m).

Topoisomerase assays

From the tumor specimens used for topoisomerase assays also cryostat sections, stained with haematoxylin-eosin, were made to compare to the paraffin embedded sections in order to assure that the specimens were representative for the tumor.

Tumor specimens (0.5 ± 0.2 cm^3) of the tumors were grossly minced with two scalpels and pulverized with a potter at 0°C. The tissue homogenate was suspended in a

small volume of nucleus buffer (0.15 M NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM dithiotreitol and 1 mM PMSF, pH 6.4) on ice. Extraction of nuclear enzymes was performed by adding an equal volume of 0.55 M NaCl nucleus buffer (final concentration 0.35 M NaCl) for 60 min on ice. Protein concentrations were determined with the method of Bradford (35). The enzyme solution was diluted with an equal volume of 87% glycerol and stored at -20°C for less than 24 hrs. Topo I activity was determined by measurement of the ATP-independent relaxation of supercoiled plasmid BR322 DNA, Topo II activity by measurement of the ATP dependent decatenation of kDNA as previously described (25). Topo I and II activity were expressed in units. One unit Topo I activity was defined as the lowest concentration of protein capable of relaxation of 0.9 µg supercoiled plasmid BR322 DNA. One unit Topo II activity was defined as the lowest concentration of protein capable of complete decatenation of 0.2 µg kDNA. Levels of Topo I and II activity in tumor extracts were compared by serially diluting extracts with the same protein concentration. All experiments were performed in duplicate. To avoid contamination by connective tissue, the epithelium of the cystadenomas and borderline tumors were dissected away from the cyst wall and used for further analysis. To correct for possibly different contamination of the tumor samples with red blood cells, which show an undetectable Topo I and II activity, the concentration of hemoglobin in all the tumor extracts was measured by a second derivative spectrophotometric assay for hemoglobin in serum (36). The percentage of protein extracted from red blood cells in the tumor could be calculated after dividing the hemoglobin concentration in the tumor extracts by the hemoglobin concentration found in a whole blood extract.

Statistics

Statistical analysis of the distribution of tumor volume index and mitotic index in the different groups was performed with the unpaired Student's t- test, for the distribution of P-gp expression and the Topo I and II activity in the different groups the Wilcoxon's rank sum test was used. Frequency tables were analyzed using the chi-square test with Yates's correction for small numbers. Rank correlations were calculated by the method of Spearman. Only p- values < 0.05 were considered significant.

Results

Tumor pathology, mitotic index, and tumor volume index

Six patients had benign cystadenomas, two patients borderline disease, 21 patients untreated ovarian cystadenocarcinomas, six patients residual disease at second look operation after chemotherapy, and seven patients recurrent disease after pathologically confirmed complete remission. For specification of chemotherapeutic regimens in these patients, see table 1.

Table 1 Chemotherapy used in patients with residual or recurrent disease.

Patients	Chemotherapy
1 res.d.	CC (6x)
2 res.d.	CC (6x)
3 res.d.	CC (3x)
4 res.d.	CC (6x)
5 res.d.	CC (6x)
6 res.d.	CC (6x)
7 rec.d.	CHAP (6x),Cy (5x)
8 rec.d.	CC (6x)
9 rec.d.	CP (5x),CC (2x)
10 rec.d.	CC (9x)
11 rec.d.	CC (6x)
12 rec.d.	CC(6x)
13 rec.d.	CC(6x),P/Vp(3x)

Res.d., residual disease; rec.d., recurrent disease; CP, cyclophosphamide, cisplatin; CC, cyclophosphamide, carboplatin; CHAP, cyclophosphamide, hexamethylmelamine, adriamycin,cisplatin; Cy, cyclophosphamide; P/Vp, cisplatin i.v., etoposide i.p.; (nx = number of cycles).

Mean mitotic index, and mean tumor volume index are shown in table 2. No differences were found in mean mitotic index in untreated ovarian carcinoma (23.8 mitotic figures / 10 high power fields, SD: 19.7), residual disease (17.0 mitotic figures / 10 high power fields, SD: 22.2), and recurrent disease (16.6 mitotic figures / 10 high power fields, SD: 11.5). Also no significant differences were found in mean tumor volume index in untreated ovarian carcinoma (48.4%, SD: 19.9), in residual disease (52.5%, SD: 24.4), and in recurrent disease (48.5%, SD: 24.6).

Immunohistochemistry

The results of C219 immunohistochemistry are shown in table 2 and 3, and figure 1. The interobserver variability between the two pathologists was always < 5%. In 16 out of the 21 untreated ovarian carcinomas variable P-gp expression (≥ 5% C219 positive tumor cells) was found with a median % positive staining cells in the 16 positive tumors: 10.0 (range: 5 - 50). In the specimens of one patient 30% of the tumor cells in the ovary were positive, while 5% of the tumor cells in the omental metastasis were positive. In one patient 5% of the tumor cells stained positive before and after chemotherapy (Pt, Cy).

In eight out of 13 chemotherapeutically treated ovarian carcinomas P-gp expression was found (median % positive staining cells: 12.5 (range: 5 - 30)). Two patients with recurrent disease, who also received MDR related chemotherapy, showed P-gp

Table 2 Mitotic index, tumor volume index, and P-glycoprotein expression in ovarian tumors.

Tumor	MI	TVI	% C219 + cells
Benign (n=8)	-	-	0 (0 - 100)
Untr.ca.(n=22)	23.8 ± 19.7	48.4 ± 19.9	7.5 (5 - 50)
Res./Rec.(n=13)	16.8 ± 16.3	50.1 ± 24.5	5 (5-30)

Untr.ca., untreated carcinoma; Res./Rec., residual and recurrent disease; MI, mean mitotic index (mitotic figures / 10 high power fields) ± standard deviation; TVI, mean tumor volume index (percentage of malignant epithelial tissue) ± standard deviation; % C219 + cells, median percentage C219 positive staining tumor cells (range).

Table 3 Intensity and location of C219 signal in C219 positive tumors.

Tumor	C219 intensity		C219 location		
	+	++	c	c/m	m
Benign (n=3)	2	1	1	0	2
Untr.ca.(n=16)	13	3	12	3	1
Res./Rec.(n=8)	6	2	7	1	0

Untr.ca., untreated carcinoma; Res./Rec., residual and recurrent disease; for specification of C219 intensity, see materials and methods; c, cytoplasmatic; c/m, cytoplasmatic and membrane bound; m, membrane bound.

expression (one patient 10% + cells, cytoplasmatic; one patient 20% ++ cells, cytoplasmatic and membrane bound). P-gp expression, neither the percentage positive cells nor the intensity of staining, did correlate with histologic type, Topo I activity or Topo II activity. In the large majority of the C219 positive malignant tumors (19/24) the intensity of the C219 signal was comparable to the intensity of staining of the 8226DOX4-6 cell line (+). In 19 of the 24 C219 positive malignant tumors the staining pattern was cytoplasmatic.

Topo I and II activity.

Storage of the tumor specimens at -180°C for several months did not influence Topo I or II activity. Tumor extracts could not be stored at -20°C because than a significant decrease in Topo II activity occurred within several days. All Topo I and II assays of the same tumor extracts were performed in duplicate, and were highly reproducible. In two patients specimens from two different sites of the same tumor showed the same Topo I and II activity. In one patient biopsies were taken from the tumor of the ovary

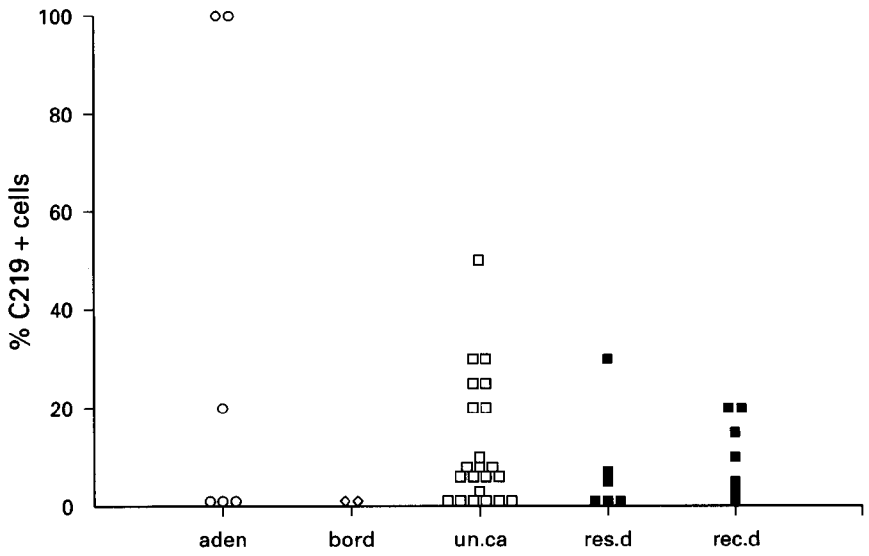

Figure 1 P-gp expression (percentage C219 positive staining tumor cells) in ovarian tumors. (aden., cystadenoma; bord., borderline disease; un.ca., untreated cancer; res.d., residual disease; rec.d., recurrent disease.

and metastatic tumor in the omentum at the same time. Topo I activity was fourfold higher in the metastatic tumor, while Topo II activity was twofold lower in the metastatic tumor. From other patients only one tumor specimen was available for Topo I and II determination. Median and range of Topo I and II activity of the different groups are shown in table 4.

Table 4 Median and range of Topo I and II activity in ovarian tumors

Tumors	no.	Topo I*	Topo II**
Benign	8	0.5 (0.4-1.6)	a
Untr.ca.	21	4.0 (2.0-16.0)	7.5 (1.0-16.0)
Res./rec.	13	4.0 (2.0-16.0)	2.0 (1.0-8.0)

*: x 104 U/mg protein; **: x 102 U/mg protein; a, detectable, but too low to quantitate; Untr. ca., untreated cancer; Res./rec., residual and recurrent disease.

For individual Topo I and II activities, see figures 2 and 3. Topo I activity in treated plus untreated ovarian carcinoma (median: 4.0 x 10^4 U/µg protein, range: 2.0 - 16.0) was higher ($p<0.01$) compared to the Topo I activity in the group of benign cystadenoma plus borderline disease (median: 0.5 x 10^4 U/mg protein, range: 0.4 - 1.6). No

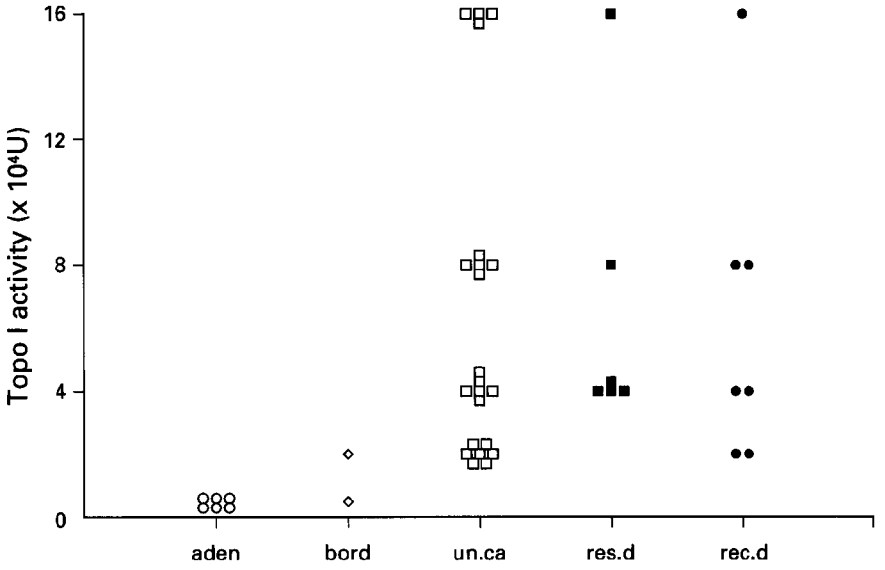

Figure 2 Topo I activity (x 10^4 U/mg protein) in ovarian tumors. (aden., cystadenoma; bord., borderline disease; un.ca., untreated carcinoma; res.d., residual disease; rec.d., recurrent disease.

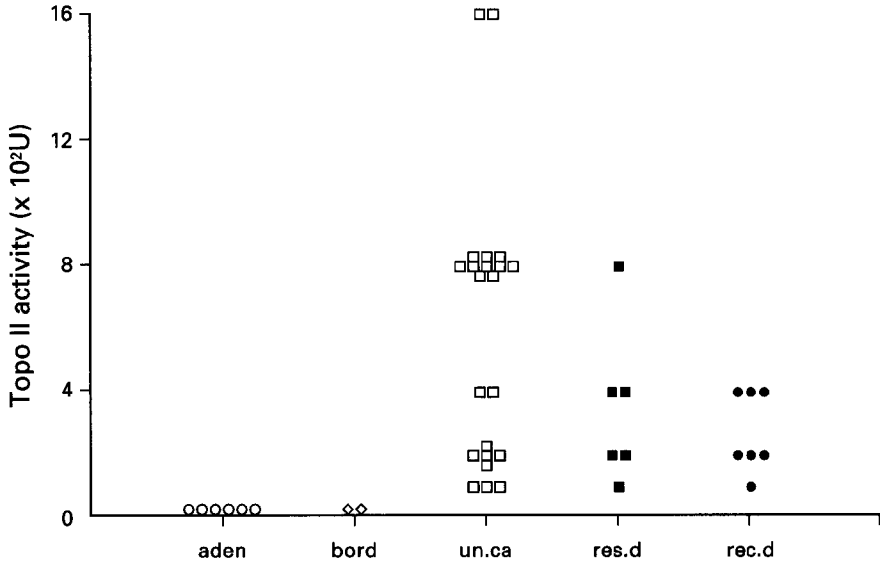

Figure 3 Topo II activity (x 102 YU/mg protein) in ovarian tumors. (aden., cystadenoma; bord., borderline disease; un.ca., untreated carcinoma; res.d., residual disease; rec.d., recurrent disease.

significant differences were found in Topo I activity in untreated ovarian carcinoma compared to Topo I activity in residual plus recurrent ovarian carcinoma. An eightfold range was found for highest and lowest Topo I activity in all malignant tumors (2 - 16 x 10^4 U/mg protein). Topo II activity was elevated in the group of treated plus untreated ovarian carcinoma (median 4.0 x 10^2 U/mg protein, range: 1.0 - 16) compared to the group of benign cystadenoma plus borderline disease (detectable, but too low to quantitate). The incidence of a high ($\geq$ 8 x 10^2 U/mg protein) Topo II activity was higher in untreated ovarian carcinoma compared to residual plus recurrent ovarian carcinoma ($p<0.05$). A sixteenfold range was found for highest and lowest Topo II activity in malignant tumors (1 - 16 x 10^2 U/mg protein). In one patient tumor specimens, taken before and after chemotherapy (cisplatin, cyclophosphamide), showed equal Topo I and II activity. Weak rank correlations were found for Topo II activity in all malignant tumors with the tumor volume index ($r = 0.35$, $p<0.05$) and for Topo II activity with Topo I activity ($r = 0.36$, $p<0.05$)(figure 4). No rank correlation was found for Topo I or II activity with the mitotic index. In the tumor extracts the percentage of protein from red blood cells in the tumor ranged from 0 - 5%. Considering these very low percentages in all the tumor extracts no correction for contamination of the tumor extracts with protein from red blood cells was performed.

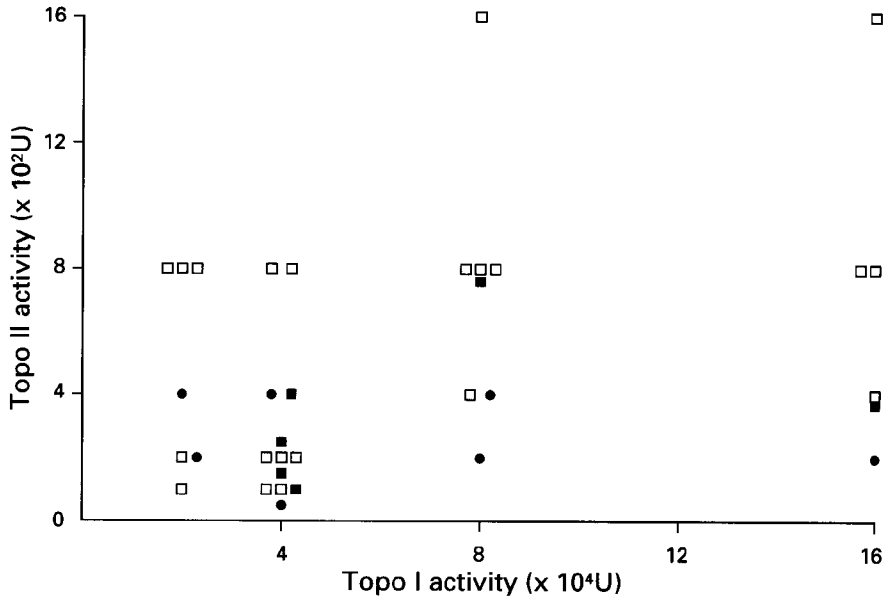

Figure 4 Topo I activity (x 10^4 U / mg protein) and Topo II activity (x 10^2 U / mg protein) in malignant ovarian tumors, $r = 0.36$, $p < 0.05$. □, untreated carcinoma; ■, residual disease; ●, recurrent disease.

Discussion

Our results show a widely differing expression of P-gp in benign as well as in malignant ovarian tumors. Malignant tumors arising in organs naturally expressing high levels of P-gp are often intrinsically resistant to MDR related chemotherapy. Previously no P-gp expression was found in normal human ovaries (37,38). In our study P-gp expression was found in three of six benign epithelial ovarian tumors, and in 16 of 21 untreated malignant tumors. Our results with an indirect immunohistochemical technique are in contrast with previous studies, using Northern and slot blot hybridization techniques, in which no P-gp mRNA expression was found in untreated ovarian carcinoma (10,11). Chan et al showed in human ovarian cell lines with the MDR phenotype that immunocytochemical detection of P-gp was as sensitive as Northern blot and more sensitive than standard Western blot (16). Perhaps homogenization of biopsies of tumor tissue, required for the various types of electrophoretic analyses may produce false negative results in tumor biopsies despite the presence of small subpopulations of P-gp bearing cells. Our results are also in contrast with the results of Rubin et al, who found P-gp expression in only four of 57 patients with ovarian carcinoma. However Rubin et al used a two step immunoperoxidase technique, which provides less signal amplification as the three step immunoperoxidase technique, used in this study. Recently Noonan et al also found a relative high incidence of P-gp expression in 21 of 26 untreated malignant ovarian tumors with a sensitive detecting method, based on the polymerase chain reaction (39). Variations in detecting P-gp expression by different techniques were also found in human breast carcinoma (40,41). Our data suggest that, in contrast to earlier conclusions, previous treatment with MDR related drugs is not necessary for P-gp expression in ovarian carcinoma (12). The intensity of the C219 staining in the majority of the malignant tumors in our study was low, and located in the cytoplasma. In cell lines, lower degrees of drug resistance to natural products were associated with staining at cytoplasmatic sites, while higher degrees of resistance appeared to be associated with membrane bound staining (42). However, studies in soft tissue sarcomas and acute leukemias showed that any immunohistochemically detectable P-gp expression was clinically relevant (7,8).

Over the last years much knowledge is gathered on topoisomerases and drug resistance in cell lines, but little information exists on these nuclear enzymes in human neoplasms, especially in solid malignant tumors. Recently, Holden et al measured Topo I and II activity in six human neoplasms and nine normal tissue samples (43). In contrast to our findings, Holden et al found equal Topo I and II levels in neoplastic specimens, such as lymphomas, breast and thyroid carcinoma, and in normal nonproliferating tissues, such as spleen and small intestine. Nelson et al found in rat prostatic adenocarcinomas higher Topo I and II activity in malignant parts of the prostate than

in benign parts (44). This is in agreement with the present study in which higher Topo I and II activities in malignant ovarian tumors were found compared to benign tumors. Hsiang et al found with immunoblotting that Topo II activity in untreated colorectal tumors varied from undetectable to elevated, while Topo II activity was low in normal colorectal mucosa (45). In cultured cell lines Topo II activity is cell cycle dependent, and in resting cells there is a lower level of Topo II activity (46). In this study no correlation was found between mitotic index with Topo II levels in the malignant ovarian tumors. This may be due to a high number of resting cells in solid tumors such as ovarian carcinomas, but the mitotic index is also a relatively rough reflection of the proliferative status of a malignant tumor. However, high Topo II activity may also be an independent parameter in ovarian carcinoma. A weak correlation was found between tumor volume index and Topo II activity, while the tumor volume index did not differ between untreated and Pt/Cy treated malignant ovarian tumors. This correlation was too weak to explain the 8- and 16-fold differences in Topo I and II activity between the individual malignant tumors. Potmesil et al suggested that the low levels of Topo II found in chronic lymphocytic leukemia cells offered an explanation for the ineffectiveness of doxorubicin treatment in patients with chronic lymphocytic leukemia (46). The differences found in Topo II activity between the malignant ovarian tumors in combination with the heterogeneity in P-gp expression may offer an explanation for the varying responses to MDR drugs in ovarian carcinoma patients (2-4). The increased incidence of low Topo II activity, found in this study in Pt/Cy treated tumors perhaps explains lower response rates to Topo II targeted drugs after first line chemotherapy (4).

In cell lines conflicting data exist on the role of Topo II in resistance to platinum and alkylating agents (28-30). Most Pt/Cy treated malignant ovarian tumors in this study showed a low Topo II activity. Lower Topo II activity in Pt/Cy treated tumors can perhaps be explained by selection of tumor cells with lower Topo II activity by Pt/Cy treatment or may be reflecting a lower metabolic activity or a decreased fraction of tumor cells in S- phase in these tumors after Pt/Cy treatment.

The increased Topo I activity in malignant ovarian tumors makes Topo I an interesting target for chemotherapy in ovarian carcinoma. Giovanella et al showed that Topo I measured with immunoblotting was elevated in advanced stages of human colon adenocarcinoma and in xenografts of colon carcinoma carried by immunodeficient mice, compared to normal mucosa. A synthetic analogue of campthothecin was found to be highly effective against these xenografted human colon carcinomas with high Topo I activity levels (47).

In conclusion, our observations confirm a possible role for P-gp expression in intrinsic drug resistance to natural drugs in ovarian carcinoma. Topo I and II activity were higher in malignant ovarian tumors compared to benign and borderline tumors. Topo II activity was lower in Pt/Cy treated tumors compared to untreated tumors.

Important variability in P-gp expression and in Topo I and II activity was found in untreated and Pt/Cy treated malignant ovarian tumors. Because of the increased Topo I activity in all malignant ovarian tumors, drugs targeted at the Topo I enzyme could be interesting for further studies. Determination of P-gp expression, as well as Topo I and Topo II activity might help in the future to select the proper treatment for the individual patient.

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Chapter 4

Quantitative and qualitative aspects of topoisomerase I and IIα and β in untreated and platinum/ cyclophosphamide treated malignant ovarian tumors

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Abstract

Quantitative and qualitative aspects of Topo I and II were studied in 17 malignant ovarian tumors (eight untreated and nine after Pt/Cy chemotherapy). Median Topo II catalytic activity was lower ($p<0.05$) in tumors after Pt/Cy chemotherapy in comparison to untreated tumors, while no differences were found for Topo I catalytic activity in tumors before and after chemotherapy, as was also found in a previous study, (Van der Zee et al Cancer Res., 51: 5915, 1991). VM-26 induced cleavable complex formation correlated ($r=0.60$, $p<0.05$) with Topo II activity, while Topo II decatenation activity was equally, but incompletely inhibited by VM-26 in all tumors. No differences were found in Topo II cleavage sites patterns in pBR322 DNA for all tumors using an indirect end-labeling procedure. Cleavable complex formation of Topo I by Cpt did not correlate with Topo I catalytic activity, while Topo I catalytic activity could equally and completely be inhibited by Cpt. By Western blotting Topo IIα protein expression was detected in four of eight untreated tumors and three of nine tumors after Pt/Cy chemotherapy, whereas in all tumors a 150-kDa degradation product of Topo IIß was detected. Topo I protein was detected in all tumors at varying levels, but the protein levels did not correlate with Topo I catalytic activity or cleavable complex formation by Cpt. Our study shows that Topo I and II, isolated from human malignant tumors, can be stimulated by Cpt and VM-26 respectively to induce DNA cleavage, which suggests that topoisomerases are real targets for chemotherapy in patients with ovarian cancer. From in vitro data from the literature it appears that the cleavable complex assay reflects both quantitative and qualitative changes, as well as changes in the phosphorylation state of Topo I and II. In combination with the

feasibility of the cleavable complex assay for Topo I and II in human malignant tumors, which was found in the present study, it appears that at present the determination of cleavable complex formation by tumors seems to be the most promising parameter of Topo I or II expression in human tumors to be related to response to Topo I or II targeted chemotherapy.

Introduction

Treatment success in ovarian cancer is limited by the presence of advanced disease in the majority of patients at the time of diagnosis and by the presence or development of resistance in tumor cells to available drugs (1). Compounds such as cisplatin and classical alkylating agents, which produce cytotoxicity by binding to DNA, are used in first and second line chemotherapy in ovarian cancer in combination with other drugs e.g. antracyclines and epipodophyllotoxines (2,3). Drugs, such as antracyclines, epipodophyllotoxines, and mitoxantrone have Topo II as target, while Cpt and Cpt derivatives target Topo I. Topo I and II are nuclear localized enzymes involved in various DNA transactions such as replication, transcription, and recombination (4). In tumor cell lines selected for resistance to the cytotoxic effects of Topo II targeted drugs multiple mechanisms can account for resistance including overexpression of the drug extrusion pump P-gp (5), and quantitative and/or qualitative changes in Topo II. Several in vitro studies have shown, that, apart from decreased Topo II levels, qualitative alterations in Topo II, caused by mutations in the Topo II gene, can lead to insensitivity of this drug target (6,7).

Recent studies have demonstrated the presence of two isozyme forms of Topo II, a 170 kD enzyme (Topo IIα) and a 180 kD enzyme (Topo IIß), which are the products of two different genes (8). In tumor cell lines these two isozymes appear to have different sensitivity to several antineoplastic drugs. Topo IIα was found to be three-fold more sensitive to VM-26, and eight to ten-fold more sensitive to merbarone than Topo IIß. Therefore both the levels and ratio of Topo IIα and ß may be important factors in determining sensitivity of tumor cells to Topo II directed drugs (9-12).

In vitro regulation of Topo II catalytic activity in tumor cells has been found to be different in comparison to normal proliferating cells, and appears to be dependent on both growth state and transformation state of the tumor cells. Topo IIα protein expression increases during S and G2 phases, peaks in G2/M, and decreases in G1 phase, while Topo IIß protein expression is approximately constant throughout the cell cycle (12-14).

Limited data do exist on topoisomerases in human solid tumor specimens (15-22). In a previous study we found higher Topo I and II catalytic activity in malignant ovarian tumors in comparison to benign tumors, no changes in Topo I catalytic activity before

or after Pt/Cy chemotherapy, but lower Topo II catalytic activity in tumors after Pt/Cy chemotherapy in comparison to untreated tumors (19). In the present study Topo I and II inhibition and cleavable complex assays, and analysis of Topo II cleavage sites were used to investigate if, in addition to quantitative differences, qualitative differences exist in Topo I and II in malignant ovarian tumors before and after Pt/Cy chemotherapy. Since the Topo II catalytic activity assay does not discriminate between Topo IIα or ß isozyme activity, Western blotting was performed to detect these isozymes, and Topo I protein levels. In addition DNA flow cytometry was performed to relate Topo II catalytic activity and Topo IIα and ß protein expression levels to the distribution of cell cycle phases in the tumors.

Materials and methods

DNA, and chemicals

Form I kinetoplast DNA was isolated as described previously (19) and supercoiled dimer of plasmid BR322 DNA, prepared from Escherichia coli strain HB 101 was a generous gift from Dr. Douwe van Sinderen, Department of Molecular Genetics, State University of Groningen, The Netherlands. VM-26 was purchased from Bristol-Meyers Co. (Troisdorf, Germany), and Cpt from Sigma Chemical Co. (St.Louis, MO). Cpt was dissolved in dimethyl sulfoxide. ATP, proteinase K, RNase, Triton X-100 and PMSF were obtained from Merck (Darmstadt, Germany). Restriction enzymes were purchased from Boehringer Mannheim (Mannheim, Germany).

Human material

Ovarian tumor specimens were obtained from patients consecutively operated at cooperating hospitals in the northern part of the Netherlands during the first five months of 1990. The tumor collection was supervised by a pathologist. The samples for topoisomerase extraction were immediately frozen in liquid nitrogen, and stored at -180°C until further analysis. Samples were divided in three parts for pathological characterization, Topo I and II assays and flow cytometry. As internal standard in the immunoblotting experiments nuclear extract of the well defined human small cell lung carcinoma cell line GLC_4 was used (23).

Pathological characteristics

The tumors were histologically classified according to the World Health Organization classification using paraffin embedded tissue sections (24). One section per cm tumor diameter was made to get a good overall impression of the tumor histology. The tumor volume index (percentage of malignant epithelial tissue in tumor specimen) was measured in the paraffin embedded sections with a point counting technique, using a

42-point grid placed on a projection microscope at a magnification of 200-fold as described by Baak et al (25).

Topo I and II catalytic activity and inhibition assays

Extraction of nuclear enzymes was carried out as described previously (19). Tumor specimens (0.5 ± 0.2 cm^3) were mechanically homogenized using a microdismembranator at 0°C. Extraction of nuclear enzymes was performed in 0.35 M NaCl nucleus buffer (1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EDTA, 0.2 mM dithiotreitol and 1 mM PMSF, pH 6.4) for 60 min on ice. Protein concentrations were determined with the method of Bradford (26). Topo I catalytic activity was assayed by the ATP-independent relaxation of supercoiled pBR322 DNA, and the Topo II catalytic activity by measurement of decatenation of kDNA in the presence of 1 mM ATP. Topo I and II catalytic activity were expressed in units, as previously described (19). Relaxation of supercoiled pBR322 DNA by tumor extracted Topo I was inhibited by rising concentrations of Cpt (0, 10, 20, 40, and 80 μM); decatenation of kDNA by tumor extracted Topo II was inhibited by rising concentrations of VM-26 (0, 5, 25, and 125 μM) (27-29). In order to provide equivalent amounts of Topo I and II catalytic activity in the different tumor extracts the amount of nuclear protein extracts was adjusted to one dilution step below the highest dilution still showing complete relaxation of 0.9 μg pBR322 DNA or complete decatenation of 0.2 μg kDNA. Topo I and II inhibition were carried out by incubating 5 μl tumor extract with 0.9 μg supercoiled pBR322 DNA or 0.2 μg kDNA and drug in a final volume of 25 μl standard reaction mixture for 30 min at 37°C. The reactions were terminated by the addition of 5 μl 3% SDS, 0.3% bromophenol blue, and 30% glycerol. All experiments were performed in duplicate.

Topo I and II cleavable complex assays

Topo I-induced cleavage of DNA was assayed by the generation of nicked (form II) DNA from supercoiled pBR322 DNA (form I) in the presence of two different concentrations (1 and 20 μM) of Cpt. Topo II-induced cleavage of DNA was assayed by the generation of form III (linearized) DNA from supercoiled (form I) pBR322 DNA in the presence of 1 mM ATP and two concentrations of VM-26 (10 and 100 μM) (27-29). For quantitation of cleavable complex formation above mentioned drug concentrations were chosen after determination of minimal and maximal effective concentrations of Cpt and VM-26 for cleavable complex formation. The standard reaction mixture for DNA cleavage was the same as described for the relaxation and decatenation assay. Topo I or II-induced DNA cleavage were performed by incubating 5 μg extracted nuclear protein of the tumors with 0.9 μg supercoiled pBR322 DNA and drug in a final volume of 25 μl standard reaction mixture for 30 min at 37°C. The reactions were terminated by the addition of 2.5 μl SDS (10%) and 2.5 μl proteinase

K (5 mg/ml), while the samples were kept at 37°C. Following an additional incubation for 30 min at 37°C, 6 µl of 0.3% bromophenol blue and 30% glycerol were added. Samples were analyzed by gel electrophoresis in 1% agarose at 20 V for 16 h in the presence (Topo I) or absence (Topo II) of 0.5 µg/ml ethidium bromide. Topo I or II-induced DNA cleavage was quantitated by scanning the photographic negatives with a LKB Ultroscan XL laser densitometer.

Mapping of Topo II cleavage sites by indirect end-labeling

To map Topo II cleavage sites on pBR322 DNA, an indirect end-labeling procedure was followed (31). DNA cleavage reactions were performed for 30 min at 37°C as described above. However, a total reaction volume of 75 µl with 2 µg pBR322, 15 µl of tumor extract and 100 µM VM-26 was used. The reaction was terminated with 7.5 µl SDS (10%) and 7.5 µl proteinase K (5mg/ml) and incubated for 3 h at 37°C. Samples were then placed on ice, and centrifuged. The supernatant was extracted with phenol/chloroform followed by ethanol precipitation. DNA samples were digested with *Eco*RI for 1 h. DNA gel electrophoresis was performed in 1% agarose gels. Southern transfer was carried out by diffusion blotting of the restriction fragments from the agarose gel on GeneScreen Plus (NEN-Dupont, Boston, MA) under alkaline conditions, according to Sambrook et al (32). Hybridization and signal detection were performed with the enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection system (Amersham, UK). The smaller fragment from *Eco*RI/*Sal*I double-digestion of pBR322 DNA was used as a probe in Southern hybridization by direct labeling with horseradish peroxidase as described by the manufacturer.

Flow cytometry

After mincing with scissors tumor samples were suspended in 0.1% RNase and 0.1% Triton X-100 solution. Lyzed cells were filtered through a nylon mesh and stained with propidium iodide. The nuclear fraction was collected and DNA histograms were generated on a Becton Dickinson FACS 440 and analyzed by using a DNA cell-cycle analysis program (Becton Dickinson Version C 12/86).

SDS-Polyacrylamide gel electrophoresis and Western blotting

Tumor extracts were prepared as described above; extracts were boiled for 5 min in equal volumes of 2x SDS sample buffer (0.5 M Tris.HCl (pH 6.8), 4% SDS, 20% glycerol, 0.002% bromophenol blue, 10% 2-mercaptoethanol). SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (33). Extracted nuclear protein (100 µg) of tumors was loaded on a 6% SDS-polyacrylamide gel. After electrophoresis proteins were transferred from acrylamide gel onto Immobilon PVDF nylon membrane (Millipore Corporation, Bedford, MA) at 200 mA for 45 min at room temperature by using a semi-dry-blot system. Topo IIα was detected using a

polyclonal rabbit antibody to human Topo IIα (1:1000), purchased from Cambridge Research Biochemicals Limited (Northwich, UK). Topo IIß was measured using a monoclonal antibody 8F8 to Topo IIß (1:200) (34), kindly provided by Dr. Giulia Astaldi Ricotti, Instituto di Genetica Biochimica ed Evoluzionistica del Consiglio Nazionale delle Ricerche, Pavia, Italy. Topo I was detected with a systemic sclerosis patient's serum (1:250) (23). ECL Western blotting systems (Amersham, UK), using protein A conjugated horseradish peroxidase, rabbit anti mouse IgG or anti human IgG as second antibody, were used to detect topoisomerases. Quantitation was performed by scanning autoradiographs with a LKB Ultroscan XL laser densitometer. As internal standard 20 μg extracted nuclear protein from the GLC_4 cell line was used in each blot.

Statistics

Statistical analysis of the distribution of tumor volume index and the different parameters of the cell cycle distribution in the different groups was performed with the unpaired Student's t-test. For the distribution of Topo I and II catalytic activity in the pre- and post chemotherapy group of patients the Wilcoxon's rank sum test was used. Rank correlations were calculated by the method of Spearman. Only p- values < 0.05 were considered significant.

Results

Tumor Pathology and Tumor Volume Index

Seventeen tumor samples were obtained from eight patients with untreated ovarian cystadenocarcinomas, and from nine chemotherapeutically treated patients; of these nine patients four patients had residual disease at second look operation after chemotherapy, and five patients had recurrent disease after pathologically confirmed complete remission. No paired samples from patients before and after chemotherapy were obtained. All patients with residual or recurrent disease had previously been treated with platinum containing chemotherapy in combination with cyclophosphamide. The mean tumor volume index was higher ($p<0.05$) in untreated tumors (73.7%, SD: 9.6) in comparison to the mean tumor volume index in tumors after chemotherapy (57.0%, SD: 20.6). Higher tumor volume index in untreated tumors was caused by the fact that most samples after chemotherapy were taken from sites in the abdominal space (e.g. omentum majus) where contamination with especially fat and connective tissue is inevitable. The contribution of the fat and connective tissue to the protein amount in the tumor extracts was checked by separately extracting samples of pure fat and connective tissue. Almost no protein could be extracted, possibly because of the relatively low amount of nuclei in these tissues in comparison to malignant tumor tissue.

Topo I and II catalytic activity

All Topo I and II assays of the same tumor extracts performed in duplicate were highly reproducible. Median Topo I catalytic activity in untreated tumors (4 (range: 2-32) x 10^4 U/mg protein) was not significantly lower than median Topo I catalytic activity in tumors after chemotherapy (8 (range: 2-16) x 10^4 U/mg protein). Median Topo II catalytic activity in tumors after chemotherapy (8 (range: 2-8) x 10^2 U/mg protein) was lower ($p<0.05$) than median Topo II catalytic activity in untreated tumors (16 (range: 8-32) x 10^2 U/mg protein)(Fig. 1). A very weak correlation was found ($r=0.35$, $p<0.05$) for Topo II catalytic activity with the tumor volume index. No correlation was found for Topo I catalytic activity with Topo II catalytic activity or tumor volume index in all tumors.

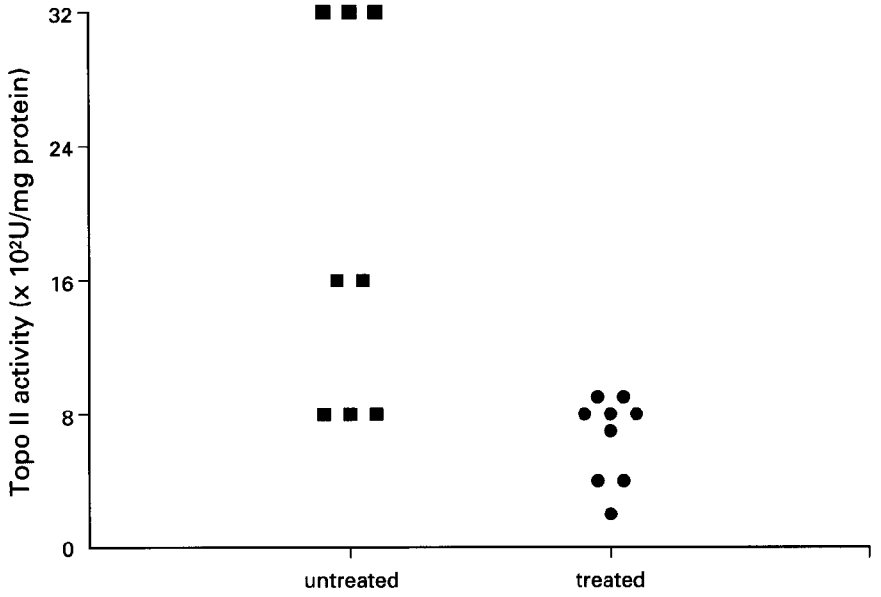

Figure 1 Topo II catalytic activity (U/mg protein) in untreated malignant ovarian tumors (■) and treated ovarian tumors after Pt/Cy chemotherapy (●).

Inhibition of Topo I relaxation activity and induction of DNA cleavage by Cpt

In all tumors Topo I catalytic activity was inhibited by raising concentrations of Cpt. Complete inhibition of equivalent (see Materials and Methods) Topo I catalytic activity by Cpt was found in all tumors at a minimal Cpt concentration of 40 μM. For cleavable complex formation minimal and optimal concentration of Cpt at the chosen extracted nuclear protein level were determined. At a Cpt concentration below 1 μM

no cleavable complex formation was found, while at concentrations higher than 20 μM formation of linear DNA interfered with the formation of nicked pBR322 DNA by Topo I. Cleavable complex formation in the presence of these two concentrations (1 and 20 μM) Cpt was found in all tumors, and was in all tumors higher at 20 μM Cpt in comparison to 1 μM Cpt. No difference was found in median levels of formation of nicked pBR322 DNA in untreated tumors in comparison to treated tumors. Formation of nicked pBR322 DNA did not correlate with the previously determined Topo I catalytic activity (Fig. 2).

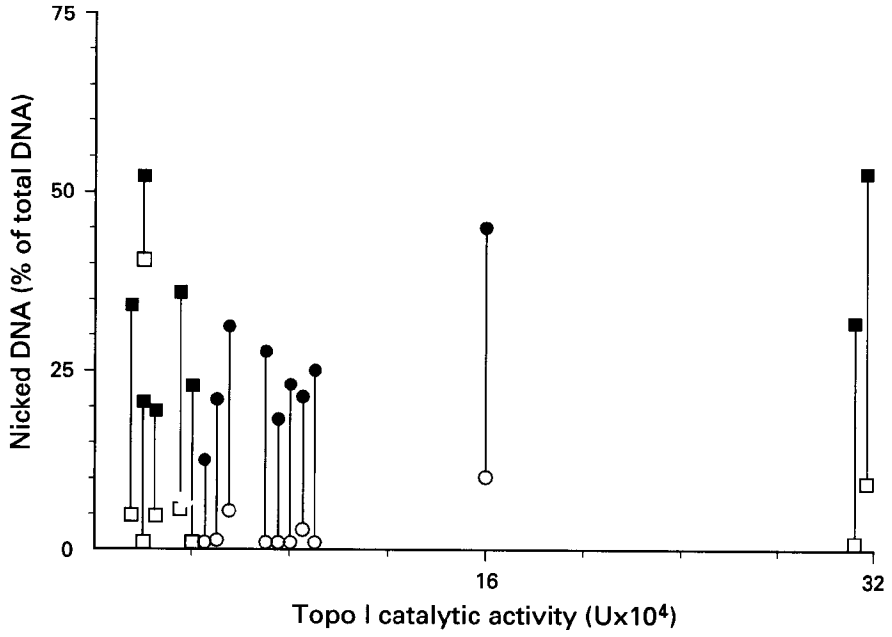

Figure 2 Relation of Topo I catalytic activity (U/mg) and cleavable complex formation by Cpt. (□ = untreated tumors, Cpt. 1 μM; ■ = untreated tumors, Cpt. 20 μM; ○ = tumors after Pt/Cy chemotherapy, Cpt. 1 μM; ● = tumors after Pt/Cy chemotherapy, Cpt. 20 μM).

Inhibition of Topo II decatenation activity and induction of DNA cleavage by VM-26

Equal, but no complete inhibition of equivalent (see Materials and Methods) Topo II catalytic activity was found in all tumors, despite using very high VM-26 concentrations (to 400 μM). Minimal and optimal concentration of VM-26 for cleavable complex formation at the chosen extracted nuclear protein level were determined. At VM-26 concentrations below 10 μM no cleavable complex formation was detectable,

whereas at VM-26 concentrations higher than 100 μM no further increase in cleavable complex formation was found. Formation of linearized pBR322 DNA by Topo II in the presence of these two (10 and 100 μM) concentrations of VM-26 was found in all tumors, and was higher in all tumors at 100 μM in comparison to 10 μM VM-26 (Fig. 3). The median level of linearized pBR322 DNA in untreated tumors (13.2, range: 5.3 - 17.9) was higher ($p<0.05$) in comparison to tumors after chemotherapy (6.2, range: 1.3 - 15.0) at 100 μM VM-26. Formation of linearized pBR322 DNA correlated weakly with Topo II catalytic activity at 100 μM VM-26 ($r=0.60$, $p<0.05$).

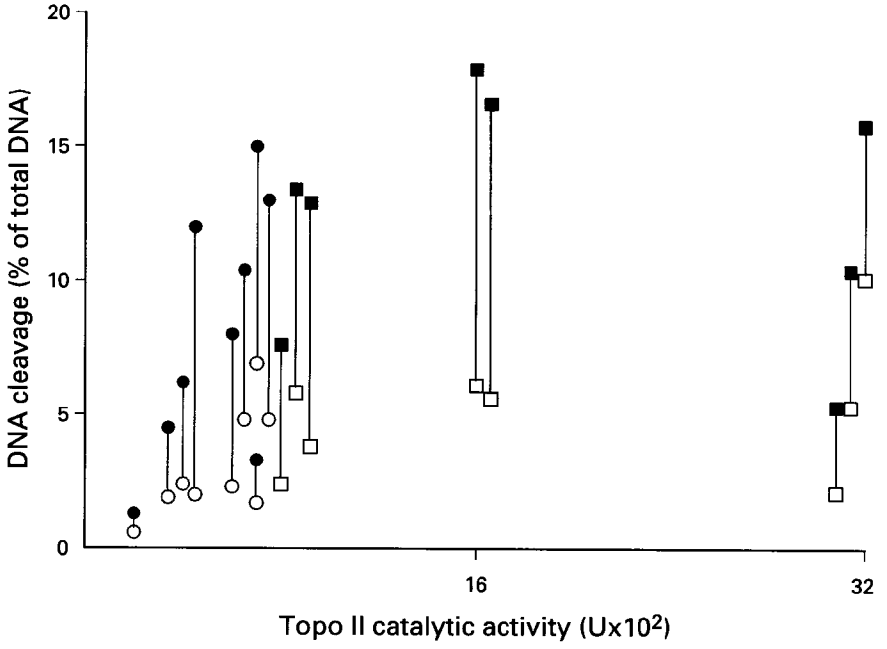

Figure 3 Relation of Topo II catalytic activity (U/mg) and cleavable complex formation by VM-26 (□ = untreated tumors, VM-26 10 μM; ■ = untreated tumors, VM-26 100 μM; ○ = tumors after Pt/Cy chemotherapy, VM-26 10 μM; ● = tumors after Pt/Cy chemotherapy, VM-26 100 μM).

Topo II cleavage sites

Fig. 4 shows characteristic pBR322 DNA cleavage sites patterns in one tumor before and one tumor after chemotherapy. No major differences were found in patterns of Topo II cleavage sites in all 17 tumors in the presence of VM-26, and therefore no indication for qualitative differences. In the absence of ATP a strong reduction in DNA cleavage sites was detected (results not shown).

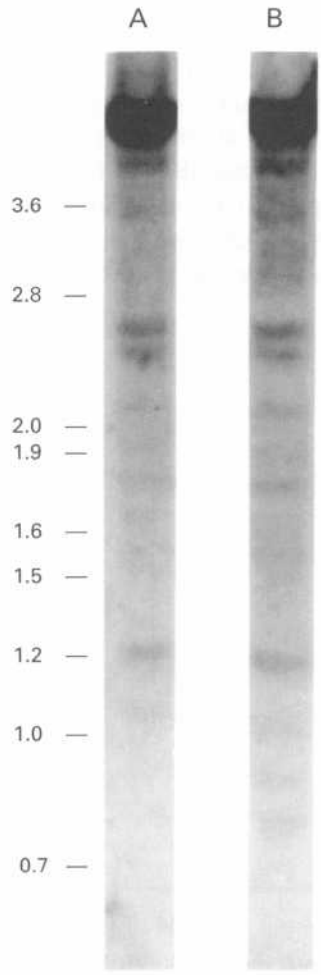

Figure 4 Mapping of *in vitro* Topo II cleavage sites on pBR322 DNA. Negatively supercoiled pBR322 DNAs were incubated with extracts of untreated (A) or treated (B) tumors and 100 mM VM-26 as decribed in "Materials and Methods". Topo II-cleaved pBR322 DNAs were purified and digested with *Eco*RI, and probed with the smaller *Eco*RI-*Sal*I fragment of pBR322 DNA which was labelled with horseradish peroxidase. A representive sample of one untreated and one treated tumor is shown. Numbers on the left indicate the positions (in kilobase) on the pBR322 map.

DNA cell cycle analysis

Cell cycle analysis in untreated and treated tumors is shown in Table 1. No differences were found in cell cycle distribution in tumor cells before and after chemotherapy. All tumors contained about 20% of cells in S-G_2M. Furthermore no relation was found for

Topo I or II catalytic activity and the distribution of cell cycle phase parameters in the tumors.

Table 1 DNA cell cycle analysis in malignant ovarian tumors.

Tumors	Mean percentage cells (% ± SD)		
	G_1 phase	S-phase	G_2M phase
Untreated (n=8)	79.0 ± 15	15.5 ± 12.0	6.8 ± 3.7
Treated (n=9)	82.3 ± 6.7	11.1 ± 5.5	6.6 ± 2.8

SD: Standard Deviation

Immunodetection of topoisomerases

When loading 100 μg of extracted nuclear protein Topo IIα protein was detected in four of eight untreated tumors and in three of nine treated tumors (Fig. 5 and 6). Fig. 5 shows, that despite unavoidable freezing and thawing procedures no important degradation products of Topo IIα were detected. Topo IIα protein was not detected in tumors with a catalytic activity level below 8 x 10^2 U/mg protein. In the seven tumors with detectable Topo IIα protein Topo II catalytic activity was not related to Topo IIα protein levels. The intertumor variability of Topo IIα protein expression ranged from undetectable expression to 12.1. No relation was found for detectable Topo IIα pro-

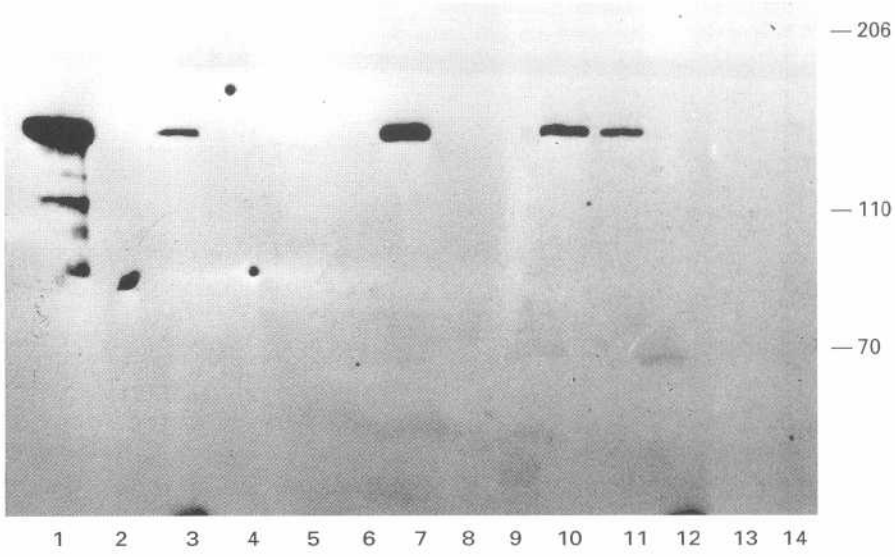

Figure 5 Topo IIα protein detection by Western blotting. Lane 1: 20 μg of extracted nuclear protein of cell line GLC_4, lane 2-14: 100 μg of extracted nuclear protein from untreated and treated tumors.

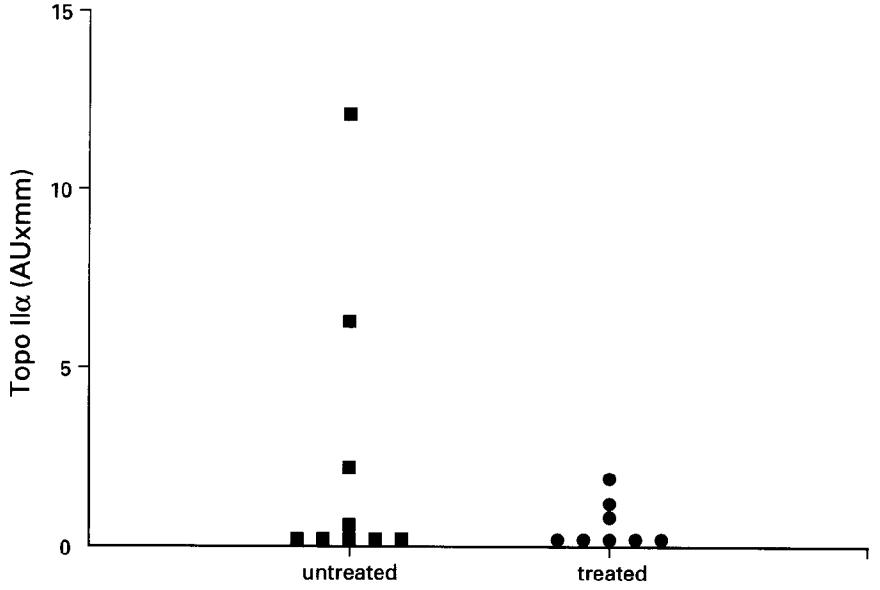

Figure 6 Topo IIα protein expression in untreated malignant ovarian tumors (■) and ovarian tumors after Pt/Cy chemotherapy (●).

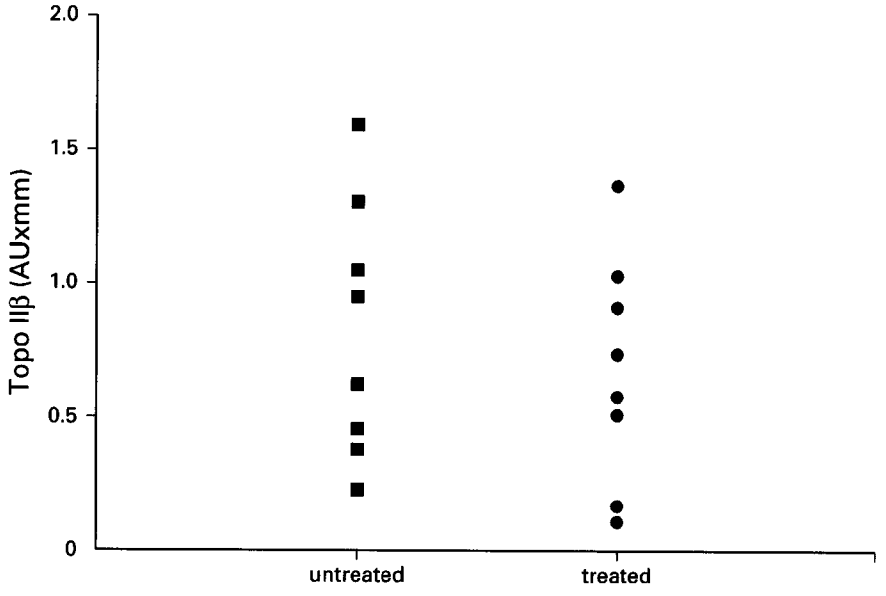

Figure 7 Topo IIβ protein expression (150-kD degradation product) in untreated malignant ovarian tumors (■) and ovarian tumors after Pt/Cy chemotherapy (●).

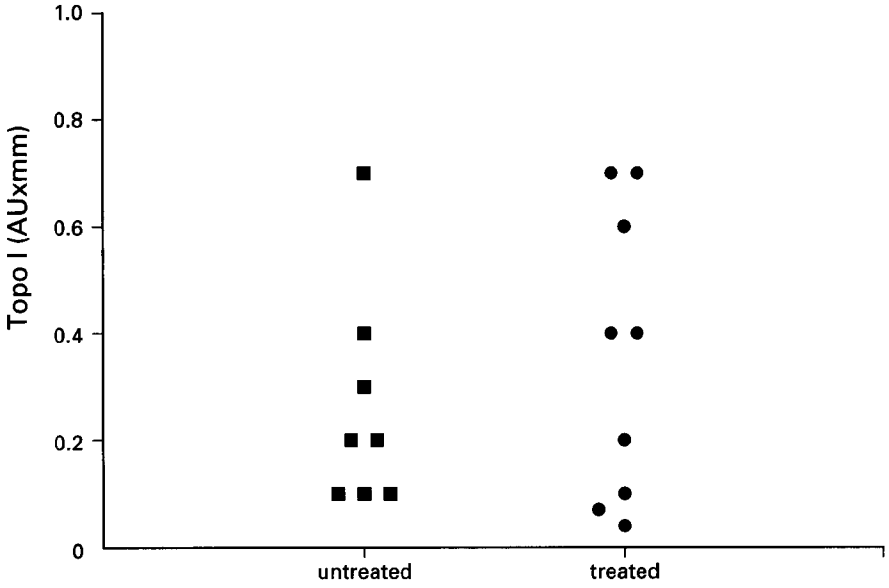

Figure 8 Topo I protein expression in untreated malignant ovarian tumors (■) and ovarian tumors after Pt/Cy chemotherapy (●).

tein levels with cleavable complex formation with VM-26, or division of cell cycle parameters in the tumors. By using the 8F8 monoclonal antibody a 150-kD protein was detected which has been identified as a degradation product of the highly unstable 180-kD Topo IIß (34). This 150-kD protein was detected in all tumors, and the intertumor variability ranged from 0.1 to 1.6 (Fig. 7). The 150-kD protein level of the tumors was not related to Topo II catalytic activity, cleavable complex formation with VM-26, Topo IIα protein levels, or distribution of cell cycle phase parameters in the tumors.

Topo I was detected in all tumors using a systemic sclerosis patient's serum when loading 100 mg nuclear protein. The intertumor variability ranged from 0.04 to 0.7. No relation was found for Topo I protein levels with Topo I catalytic activity levels or cleavable complex formation with Cpt. No difference was found in median Topo I protein expression in tumors before or after Pt/Cy chemotherapy (Fig. 8).

Discussion

Biochemical and molecular data on topoisomerases in solid human tumors are relatively rare (15-22). Furthermore, in most of these studies, only one parameter of Topo I

or II in tumors was determined such as protein expression (17,21,22), mRNA expression (18,20) or Topo II catalytic activity (19). Until now, no study has evaluated drug-induced DNA cleavage activity in tumors, although the production of stabilized Topo I- or Topo II-DNA cleavable complexes by Topo I or II targeted drugs, is thought to result in a cascade of events, that leads to cell death (4). In tumor cell lines, it was found that quantitative (35-38) as well as qualitative changes in Topo II (39-43) can be responsible for resistance to Topo II targeted drugs. The most common mechanisms thus far identified for conferring resistance to Cpt and Cpt analogues are decreased Topo I activity and/or content (22,44-46) as well as reduced sensitivity of an altered Topo I to the inhibitor (47,48). In the present study, the Topo I and II enzyme activities have been correlated with DNA cleavage activity as well as protein expression to extend our previous study. Median Topo II catalytic activity was lower and median Topo I catalytic activity similar in Pt/Cy treated tumors compared to untreated tumors confirming our previous results (19).

To evaluate the possible presence of qualitative differences in Topo II in different tumors before and after Pt/Cy chemotherapy two different biochemical assays were used, each evaluating different qualitative aspects of Topo II: DNA-cleavage by Topo II (covalent binding of Topo II to DNA in the presence of drugs) in the presence of VM-26 and inhibition of Topo II catalytic activity by VM-26 (27-30). In the presence of qualitative differences in Topo II no relation between Topo II activity of the tumor and DNA cleavage should be seen. In addition, no or a decreased inhibition of Topo II catalytic activity by VM-26 should be found. DNA cleavage activity by Topo II was observed in all tumors. To our knowledge, this study is the first to show that Topo II, extracted from human malignant ovarian tumors, which have been frozen for storage and thawn for experimental workup, can be stimulated by VM-26 to form cleavable complexes in vitro, and could thus be a real target in human malignant ovarian tumors. The comparable inhibition of Topo II catalytic activity and the approximate correlation between Topo II catalytic activity and cleavable complex formation do not point to qualitative differences in Topo II in the different tumors, before or after Pt/Cy chemotherapy. However, the cleavable complex formation varied importantly in untreated tumors with similar catalytic activity as was found in tumors with the highest Topo II activities. Therefore, further qualitative analysis of isolated Topo II was performed by mapping of Topo II cleavage sites, which did not reveal major differences in cleavage patterns in all tumors. Based on these observations no major qualitative differences seem to be present in Topo II extracted from different tumors. This may be due to the fact that qualitative differences in Topo II have only been found in cell lines selected for primary resistance to Topo II targeting drugs, while in this study tumors from untreated patients or patients after Pt/Cy chemotherapy were used.

The relative presence of the two isozymes Topo IIα and ß which differ in sensitivity for Topo II targeted drugs (9-12), may have an effect on the correlation between

catalytic activity and cleavable complex formation. The assays for Topo II catalytic activity and cleavable complex formation do not discriminate between the Topo II isozy mes. However, when Topo IIα and ß protein levels were determined using Western blotting, we still found a dissociation of Topo II catalytic activity, cleavable complex formation and Topo IIα and ß protein levels which may have several reasons. First, the relative contribution of Topo IIα and ß levels to the overall Topo II catalytic activity could not absolutely be quantified, because no isolated Topo IIα and ß were available to be used as internal standards. Second, in vitro studies showed an inverse relation of catalytic activity and phosphorylation state of Topo II with cleavable complex formation (49,50). Therefore, differences in phosphorylation of Topo II in tumors may have an effect on the relation between Topo II catalytic activity and protein levels as well as on the relation between Topo II catalytic activity and DNA cleavage activity.

In the study of Holden et al in a small series of different tumors (no evaluation of cleavable complex formation) Topo II protein was detected only in extracts of three tissues with a high percentage of cycling cells (24 to 42% of the cells in S-G_2M) (15,16), although Topo II protein levels were not related to the observed catalytic activity of Topo II in these extracts. In contrast, we found in a larger series of tumors that neither Topo II catalytic activity nor Topo IIα and ß protein levels were related to the distribution of cell cycle phase parameters in the tumors.

In this study the assays used for evaluating two different qualitative aspects of Topo I showed equal, eventually complete inhibition of catalytic activity and also cleavable complex formation by Cpt in all malignant tumors. In vitro cleavable complex formation by Cpt with Topo I extracted from malignant ovarian tumors support our previous suggestion that Topo I may be a promising target for new Topo I analogues in ovarian cancer at different stages of the disease (19). Cleavable complex formation was related to Cpt concentration, but not to Topo I catalytic activity in all tumors. Further evaluation must be awaited to confirm that the dissociation of Topo I catalytic activity and cleavable complex formation is an indication for the presence of an altered Topo I enzyme in some ovarian tumors. Unfortunately, the indirect labeling method can not be used to map Topo I cleavage sites. Another factor involved could be a difference in phosphorylation of Topo I between tumors. In contrast with the findings for Topo II Pommier et al demonstrated that phosphorylation by protein kinase C of isolated Topo I stimulated catalytic activity as well as DNA cleavage activity (51).

In panels with cell lines not selected for in-vitro resistance, both DNA cleavage activity and Topo II protein expression generally correlate with drug sensitivity, but additional factors could be involved (52-54), whereas no correlation between Topo I expression and Cpt cytotoxicity was found in a study with human lung cancer cell lines (53). A direct correlation between different parameters of Topo I or Topo II expression and drug sensitivity was observed in cell lines with acquired resistance to

respectively, Cpt or Topo II targeted drugs when compared to the parental line (43,44,49,54). However, Topo IIα and ß were not separately determined in these studies which could have an effect on the observed correlations.

In our study the greater sensitivity of the Topo I and II catalytic activity and cleavable complex assays in comparison to Western blotting is clearly shown by the fact that in these assays only 5 µg extracted protein was needed, while in the immunoblot assay at least 100 µg extracted protein had to be used. The greater sensitivity of the assay in our study in human tumors and the fact that in vitro studies show that the cleavable complex assay reflects quantitative and qualitative changes, as well as changes in phosphorylation state of Topo I and II, suggest that at present the determination of cleavable complex formation by tumors is the most promising parameter of Topo I or II expression in human tumors to be related in further studies to response to chemotherapy. Only recently the first data in different malignancies became available on the relation of Topo II mRNA levels and response to Topo II targeted drugs (18,55). In these studies the relation of Topo II mRNA levels with Topo II catalytic activity or cleavable complex formation was not evaluated.

Although the assays, as used in this study, provide quantitative and qualitative information on topoisomerases in a solid human malignancy, there are some limitations. The presence of phosphorylated topoisomerases can not be accurately determined in tumor tissue, since ^{32}P-radioactive labeling is necessary. Possibly, an estimation can be made by measuring the catalytic activity in extracts before and after dephosphorylation of the extracts. The ratio of Topo IIα and ß could not absolutely be quantified, because the anti-Topo IIß antibody is not specific and reacts with the 150-kD stable degradation product of the highly unstable Topo IIß protein. Furthermore, especially in solid tumors, such as ovarian cancer, heterogeneity of cell-biological factors, may be missed by homogenization. The development of in situ methods such as immunohistochemistry using monoclocal antibodies against Topo IIα and ß (56) or Topo IIα and ß mRNA in situ hybridisation will provide information concerning the heterogeneity of Topo II mRNA and protein levels in individual tumors.

In conclusion, our study shows that Topo I and II, isolated from human malignant ovarian tumors, are able to form cleavable complexes, which are thought to be involved in the cytotoxic effect of Topo I or II targeted drugs. Our findings also indicate that the multidrug resistant phenotype, which is commonly found in malignant ovarian tumors after Pt/Cy chemotherapy, may at least partly be due to lower Topo II catalytic activity, which is also indicated by lower overall cleavable complex formation by VM-26 in tumors after Pt/Cy chemotherapy. No indications for structural changes in Topo II enzymes were found. Our results suggest that at present the determination of cleavable complex formation is the most promising parameter of Topo I or II expression in human tumors to be related to response to chemotherapy in further studies.

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Chapter 5

Molecular analysis of the topoisomerase IIα gene and its expression in human ovarian cancer

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Abstract

DNA topoisomerase (Topo) II enzymes are key targets for the group of anti-tumor agents known as Topo inhibitors and the levels of Topo II expression in tumor biopsies may therefore predict response to treatment with enzyme inhibitors. We have used methodology which allows the sequential extraction of protein and genomic DNA from the same biopsy sample to study the Topo IIα locus and the expression in ovarian cancer. Thus, both gene expression and genetic analysis was carried out without the need to process separate pieces of tissue. This approach makes efficient use of small biopsy samples and importantly, allows data on gene expression to be correlated directly with genetic changes. Topo IIα expression was analysed in 54 tumors which is the largest series of tumors of any one particular type examined to date. Topo IIα expression was detected in 65% of ovarian tumors with a 16 fold range in level. Topo IIα expression was significantly higher in high grade and advanced stage tumors. In adenocarcinomas, the Topo IIα gene can become amplified due to its proximity to ERBB2 on chromosome 17q. Of 86 ovarian tumors studied only 1 had amplification of ERBB2 and none had amplification of Topo IIα sequences.

Introduction

The nuclear-localised Topo II enzymes are key constituents in a variety of events involving DNA metabolism such as chromosome replication and recombination (1). However, it is their identification as targets for numerous anti-tumor agents including etoposide, mAMSA and doxorubicin, which makes them of interest to study as determinants of cellular sensivity to chemotherapy (2). During the normal catalytic cycle

of the enzyme, a transient gap in the DNA is generated through which another strand of DNA can pass (3). Many of the Topo II poisons stabilise the enzyme during this stage of the catalytic cycle leading to what is termed the cleavable complex (4). The inhibition of the normal Topo II catalytic activity results in cell death (4). In vitro tissue culture models demonstrate that cells expressing high levels of Topo II are more sensitive to the cytotoxic effects of the Topo II poisons than those with low levels (2,5,6,7). Cell lines selected for resistance to Topo II poisons can become refractory to inhibition by reducing levels of expression or through mutation in the enzyme (2).

Thus, measuring levels of Topo II expression in tumors may indicate the relative sensitivity to Topo II inhibitory agents (2,7). However, despite our wealth of knowledge of Topo biochemistry and many well characterised cell lines there is still relatively little data on Topo II expression in human tumors (8,9,10,11). Recently, we determined the activity of Topo II in 43 ovarian tumors by biochemical assay and found a 16 fold range in activity between samples (8). These data suggest that Topo II is a potential target for cytotoxic therapy in ovarian cancer (8). In patients with ovarian cancer, Topo II poisons such as doxorubicin and etoposide are widely used in first and second line chemotherapy in combination with platinum (12). However, the human genome codes for both Topo II α and ß, (13,14). The isoenzymes are differently regulated during the cell cycle and possibly occupy separate sub-compartments within the nucleus, although both can be inhibited by Topo II interactive agents (15,16,17). Both isoenzymes are detected in the biochemical assay for Topo activity and are not distinguished from each other (17). Due to the differential regulation of expression of the Topo IIα and ß isoenzymes it may be of value to investigate the expression of each isoform individually to assess the relative potential of each isozyme to sensitise cells to Topo II poisons. In addition, due to localisation of Topo IIα and ß to separate chromosomes (13,14), independent carcinogenic events involving these chromosomes may lead to altered expression of either or both isozymes (18,19).

Tumor cells are known to accumulate characteristic genetic defects during their progression (20,21,22). For example, the ERBB2 oncogene which is situated on chromosome 17q21-22, is amplified in 10-30% of adenocarcinomas of the breast (21,23). The Topo IIα gene is also localised to chromosome 17q21-22 (13). It has recently been shown both in breast cancer biopsies and in an adenocarcinoma cell line which carries an amplified ERBB2 gene that the Topo IIα gene can be coamplified with ERBB2 due to physical linkage of the two genes on chromosome 17q (18,19). As a consequence of coamplification, expression of the Topo IIα gene can be increased, suggesting that tumors carrying an amplified Topo IIα gene may be sensitive to Topo II poisons (18,19). There are many similarities between breast and ovarian cancer in terms of molecular changes associated with tumorogenesis (20,21,22,23,24). Of particular relevance to Topo IIα expression is the reported amplification of ERBB2 in 20-30% of ovarian tumors (20,25,26,27,23). In addition, the long arm of chromosome 17

on which Topo IIα is located, frequently shows loss of heterozygosity in ovarian tumors and is the site of an as yet unidentified locus involved in familial breast and ovarian cancer (20,24,28,29,30).

In the current study, we have carried out a detailed molecular analysis of the Topo IIα gene and its expression in ovarian cancer. We have examined 54 ovarian tumor biopsies taken either at presentation or after therapy with platinum and cyclophosphamide for expression of Topo IIα. Topo IIα expression was determined by Western blot analysis and was detected in 65% of samples with a 16 fold range in expression between samples. Interestingly higher levels of Topo IIα expression were associated with higher grade and advanced stage tumors. In addition, the Topo IIα locus was examined in order to discover whether the expression of Topo IIα was influenced by gross genetic change. In contrast to studies on breast cancer (19), Topo IIα gene amplification was not observed and did not therefore explain the range in Topo isoenzyme expression. The lack of Topo IIα amplification in ovarian cancer is due to our observation that the ERBB2 locus is amplified at a low frequency of one out of eighty six samples.

Materials and methods

Tumor biopsies

Biopsies of ovarian tumors were collected from cooperating hospitals in the northern region of The Netherlands from 1990-1991. Tumor samples were collected at operation. After excision tumors were immediately stored in liquid nitrogen. All samples were examined histologically to confirm the presence of tumor cells. The clinical and pathological data for the patients is shown in Table 1. All patients were staged according to the International Federation of Obstetrics and Gynaecology (FIGO) classification. The tumors were histologically classified according to the World Health Organisation classification using paraffin embedded tissue sections (31). Tumors were graded in three classes: well (I), moderately (II) and poorly differentiated (III) (32). Only tumor specimens with a tumor volume index of at least 50% were included in the study. Tumor volume index is the percentage of malignant epithelial tissue in a specimen and was measured by a point counting technique, using a 42-point grid placed on a projection microscope (33).

Extraction of DNA and protein

Both protein and high molecular weight genomic DNA were sequentially extracted from biopsy samples as previously described (19). Briefly, a protein extract containing Topo enzymes was obtained by incubation of powdered tumor in 0.35M sodium chloride buffer (8). After centrifugation, the supernatant which contains the Topo

Table 1 Clinical and pathological data

Sample	TVI	Stage	Grade	hist	Topo IIα
1	75	III	II	ac	8
2	85	III	III	sac	9
3	50	III	II	ac	0.6
4	90	IV	III	ac	5.6
5	70	IV	II	sac	5.1
6	90	III	II	psac	0
7	90	III	II	psac	0
8	80	III	I	cc	0
9	50	II	I	psac	1
10	50	III	II	ac	7.7
11	50	III	II	psac	0
12	80	IV	II	psac	12.7
13	60	Ib	II	sca	0
14	80	III	III	sca	6
15	50	III	I	psac	0
16	90	IV	III	eac	15.6
17	80	III	III	ac	4.3
18	70	IV	III	ac	3.1
19	70	III	II	sca	0
20	50	III	III	ac	4.4
21	80	III	III	ac	7.1
22	50	III	II	psac	0
23	100	III	II	psac	0
24	50	Ia	I	mca	2.0
25	65	Ib	II	eac	6.5
26	80	IV	II	sca	4.9
27	75	III	II	spca	1.4
28	50	III	III	ac	2.1
29	75	III	III	ac	1.6
30	90	III	II	sca	1.0
31	80	III	II	spac	2.9
32	50	II	I	cc	0
33	85	III	II	sca	6.2
34	50	III	II	spac	0.2
35	70	III	II	spac	2.0
36	95	III	III	spac	0
37	50	III	II	sca	0.1
38	50	III	II	ac	0
39	80	IV	II	sca	0.3
40	80	III	II	sca	8.4
41	95	III	II	sca	1
42	70	III	II	sca	1
43	50	III	II	ac	1.2
44	50	III	III	ac	0
45	80	III	II	sca	9.2
46	60	III	II	sca	0
47	50	III	II	sca	4.7
48	50	III	II	sca	2.3
49	80	III	II	sca	0
50	50	III	II	sca	0
51	50	III	II	sca	0
52	80	III	II	spac	0
53	80	III	II	sca	0
54	70	III	II	sca	6.8

TVI is the tumor volume index. Histopathological type, ac: adenocarcinoma spac: serous papillary adenocarcinoma, cca: clear cell adenocarcinoma, mca: mucinous cystadenocarcinoma, eac: endometroid adenocarcinoma. Topo IIα is the level of Topo IIα expression. Samples 1-42 are presentation samples, 43-54 are post-therapy samples.

enzymes was removed and stored at -70^{0}C until use. The pellet contains the genomic DNA.

Western blot analysis

Topo IIα expression was analysed by Western blot as previously described (19), using a commercially available polyclonal anti-serum from Cambridge Research Biochemicals, Cheshire UK. Antibody complexes were visualised non-isotopically by chemiluminescence (Amersham UK). Quantitative levels of Topo IIα expression were obtained using a Molecular Dynamics laser scanning densitometer. Protein extract from the cell line GLC_4 (34) was used as a standard on all blots and was used to compare results between blots and standardise densitometry measurements.

Southern Blot analysis

Genomic DNA was extracted from ovarian tumors for analysis by Southern blotting. DNA was digested with restriction endonuclease according to the manufacturers instructions, fractioned by agarose gel electrophoresis and transferred to nylon filters. Hybridisations were carried out as previously described (18). The clone SP1 was used to detect Topo IIα sequences (35), pCER204 to detect ERBB2 (36), sequences and pHJi (immunoglobulin heavy chain) to control for loading of the gels (37).

Statistical analysis

Topo IIα expression and frequency of heterozygosity at the Topo IIα locus was analysed using chi square and Mann-Whitney tests with a 5% level of significance.

Results

Expression of Topo IIα in presentation and post-therapy samples

A collection of 54 ovarian tumors were analysed for expression of Topo IIα by Western blot (Table 1). Table 2 shows that of the 54 samples, 35 (65%) had detectable levels of α expression. The biopsy collection comprised of untreated tumors and samples from patients who had received therapy with both platinum and cyclophosphamide. The difference in frequency of Topo IIα expression between biopsies taken prior to and post-chemotherapy is not significant, using chi square analysis and a 5% level of significance. Levels of Topo IIα enzyme expression was therefore quantified by laser densitometry. Figure 2 shows the levels of Topo IIα expression in individual ovarian tumor biopsies taken either pre- or post-treatment with platinum and cyclophosphamide. Statistical comparison of the expression values pre- and post-chemotherapy using the Mann-Whitney test show no significant difference between the two groups. The combined group of tumors show a 16 fold range in expression. In two

patients tumor samples were obtained before and after chemotherapy. In one patient no Topo IIα protein was detected in the pre- or post-therapy samples. In the biopsies taken from the other patient, the pre-chemotherapy sample had detectable levels of Topo IIα expression, (level 5.6), whereas expression was undetectable in the post-chemotherapy sample.

Table 2 Topo IIα expression in ovarian tumors

Chemotherapy	[a] No. of tumors	[b] No. expressing Topo IIα
Pre	42	30 (71%)
Post	12	5 (42%)
Total	54	35 (65%)

Biopsies of ovarian tumors either pre or post chemotherapy were analysed for expression of Topo IIα by Western blot. [a]) The number of tumors examined. [b]) The number of tumors which were positive for Topo IIα expression.

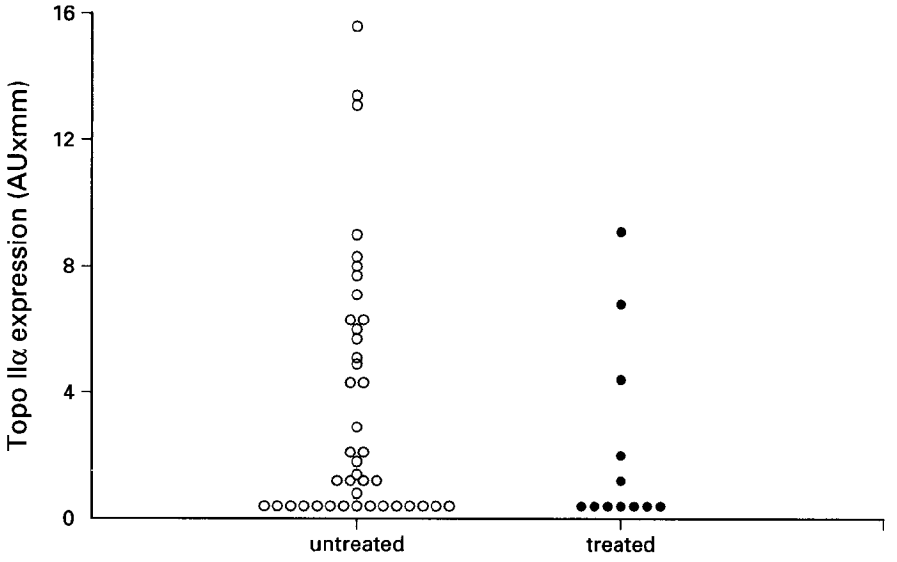

Figure 1 Range of Topo IIα expression in ovarian tumors. Levels of Topo-IIα expression was quantified by densitometry as described in Materials and Methods. The tumor population is divided into pre-treatment and post-treatment with platinum and cyclophosphamide.

Expression of Topo IIα in relation to tumor stage and grade

Topo IIα expression was analysed in relation to the grade and stage of the untreated

tumors (Table 3). For the statistical analysis using the Mann-Whitney test (Table 3b), only the untreated tumors were used to avoid possible influences of chemotherapy. As shown in tabel 3b, stage IV tumors had significantly higher levels of Topo IIα than stage III tumors (p=0.03) and stage IV tumors had significantly higher levels of Topo IIα than the combined stage I and II tumors (p=0.02). Grade III tumors had significantly higher levels of Topo IIα than grade I, (p=0.04) and grade II tumors (p=0.03). In addition grade III tumors had significantly higher levels of Topo IIα expression than the combined grade I and II tumors (p=0.02).

Genetic Analysis of the Topo IIα Locus

In order to determine whether amplification of the Topo IIα gene was responsible for the high levels of expression observed in some samples, genomic DNA was prepared from the biopsies as described in the materials and methods and Southern blot analysis carried out. Of the 54 samples for which expression data was obtained, genomic DNA of sufficient quality to analyse by Southern blotting was extracted from 51 samples. DNA from an additional 35 ovarian tumors was prepared in order to expand the genetic analysis to a group of 86 tumors. None of the 86 tumors had amplification of the Topo IIα gene. Of the 86 tumors, 1 had amplification of the ERBB2 gene and the Topo IIα gene was not co-amplified. Figure 2 shows a Southern blot of DNA extracted from ovarian tumors (lanes 2-18), the adenocarcinoma cell line CALU3, which has co-amplification of ERBB2 and Topo IIα (18) (lane 1) and normal white blood cells (lane 19). The blot shown in figure 2 was sequentially probed to detect Topo IIα (a), and ERBB2 (b). Differences in DNA loading were controlled for by hybridisation with an immunoglobulin locus probe pHJi (figure 2c). As can be seen from figure 2, the DNA extracted from the ovarian tumor analysed in lane 10 has amplified ERBB2 sequences but does not have co-amplification of Topo IIα.

Table 3a Statistical analysis of Topo IIα expression in relation to stage and grade

	N	Mean	Median	Stdev.
Stage I	5	1.9	1	2.7
Stage III	30	2.5	1	3
Stage IV	7	6.8	5.1	5.4
Grade I	3	0.7	0	1.2
Grade II	28	2.5	1	3.5
Grade III	11	5.4	4.4	4.3

Table 3b Statistical analysis of Topo IIα expression in relation to stage and grade using the Mann-Whitney test

	Tumour group 1	P	Tumour group 2
	Stage I	0.8	Stage III
	Stage I	0.10	Stage IV
	Stage III	0.03	Stage IV
Combined	Stage I/III	0.02	Stage IV
	Grade I	0.35	Grade II
	Grade I	0.04	Grade III
	Grade II	0.03	Grade III
Combined	Grades I/II	0.02	Grade III

Table 3a shows the mean and median levels of Topo IIα expression in groups of tumours sub-divided by stage and grade. Table 3b shows the p-values (p) from Mann-Whitney tests comparing Topo IIα expression between groups of tumours categorised by grade and stage. Pairwise comparisons were made between the groups of tumour shown in columns 1 and 2. The p-value obtained (p) as shown between the columns.

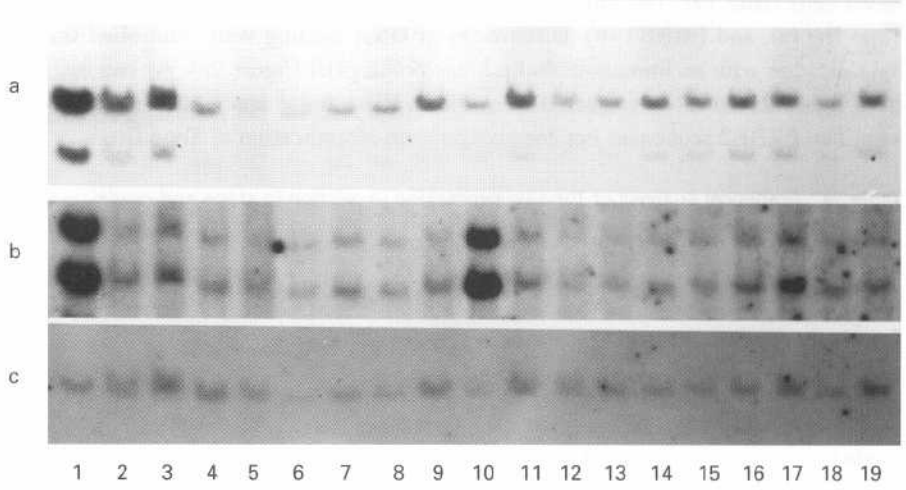

Figure 2 Southern blot analysis of the Topo IIα locus. DNA extracted from ovarian tumors was digested with Pst1 and analysed by Southern blotting. Lane 1, CALU3 cell line. Lanes 2-18, ovarian tumors. Lane 19, normal white blood cell DNA (a) hybridisation to Topo IIα sequences; (b) hybridisation to ERBB2 sequences; (c) hybridisation to immunoglobulin sequences to control for loading.

Restriction fragment length polymorphisms

Restriction fragment length polymorphisms (RFLP) can be used to detect allelic imbalances at genetic loci (19). There is an Xmn1 restriction length polymorphism at the Topo IIα locus. (18,19,38) and the frequency of heterozygosity in normal tissue samples is known to be 22%, (see reference 19 and table 4). DNA extracted from ovarian tumors was analysed for frequency of heterozygosity at the Topo IIα locus. Figure 3 shows a Southern blot of DNA extracted from ovarian tumors and digested

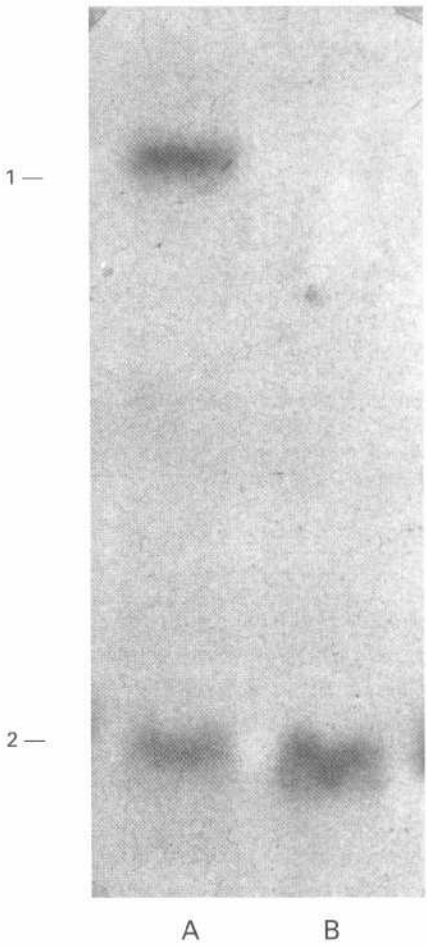

Figure 3 RFLP analysis of Topo IIα gene. DNA extracted from ovarian tumors was digested with Xmn1 and analysed by Southern blotting. The two allelic fragments 1 and 2 are shown to the left of the autoradiograph. Sample (A) is heterozygous, sample (B) is homozygous for allele 2.

with Xmn1. The two alleles detected by Xmn1 are shown in Figure 3, lane A and so the sample in lane A retains heterozygosity at the Topo IIα locus. The sample in lane B is homozygous for allele 2. Table 4 shows the frequency of heterozygosity at the Topo IIα locus in ovarian tumors pre- and post-chemotherapy. A deviation in frequency of heterozygosity in the tumor population in comparison to the normal population infers genetic change in the form of allele loss at the locus. However, no significant differences in the frequency of heterozygosity between the pre- and post-chemotherapy groups or between pre, post or combined pre and post frequencies and the frequency in the normal population. However, it is of interest that the ovarian tumor which has amplified ERBB2 sequences (Figure 2 lane 10) which originate from chromosome 17q still retains heterozygosity at the Topo IIα locus on chromosome 17q (figure 3, lane A).

Table 4 Frequency of heterozygosity at the Topo IIα locus

Sample	No [a]	Heterozygosity [b]
Normal [c]	92	20 (22%)
Pre	50	7 (14%)
Post	21	2 (9.5%)
Total	71	9 (12.7%)

DNA extracted from ovarian tumors either pre- or post chemotherapy were analysed for frequency of heterozygosity at the Topo IIα locus. [a]: The number of samples studied. [b]: Number of samples showing heterozygosity. [c]: Data from Keith et al 1993 (19).

Discussion

We have applied methodology which allows for the extraction of both protein and genomic DNA from the same sample to biopsies of ovarian cancer. This approach permits both gene expression and genetic analysis to be carried out without the need to process separate pieces of tissue and is therefore an efficient use of small tissue samples. Importantly, data on gene expression can be directly correlated with genetic change.

The expression of Topo IIα was analysed in 54 ovarian tumors using a commercially available antibody and found it to be expressed in 65% of samples with a 16 fold range in expression. This is the largest series of tumors of one particular type to be analysed for Topo IIα expression to date. Topo II poisons are valuable anti-cancer agents and the characterisation of tumors for expression of Topo II may help identify tumor types which benefit from treatment with TopoII-inhibitory drugs. A previous study of Topo II activity in ovarian cancer as determined by biochemical assay found there to be a 16 fold range in activity with all tumors having detectable levels of activ-

ity (8). In agreement with the previous study, a sixteen fold range in Topo IIα protein expression was detected. However, Topo IIα expression was detected in 65% of tumors by Western blot analysis in comparison to the previous study in which all malignant tumors had detectable Topo II activity (8). In addition, it was shown (8) that a group of untreated tumors had a significantly higher number of samples which expressed high levels of activity than a group of tumors treated with combined platinum and cyclophosphamide, whereas we found no significant difference in the frequency or levels of Topo IIα expression pre- and post-therapy with platinum and cyclophosphamide. Neither cyclophosphamide nor platinum are Topo II inhibitors and so tumors surviving treatment with these drugs would not be expected to have reduced levels of Topo II expression in order to become resistant to the cytotoxic effects of these agents (2). These differences to the previous study may be due to the methodology for analysis. In particular, the biochemical assay can potentially detect both the α and ß isoenzymes (14) whereas in the present study we have concentrated on quantifying Topo IIα expression. It is of interest however, that higher levels of Topo IIα expression are associated with stage IV and grade III tumors (table 3b). The reasons for this association are not clear but may be a reflection of the more agressive growth of the later stage and higher grade tumors. These data suggest some worth in re-evaluating the treatment of advanced ovarian tumors with therapy regimes including Topo II inhibitory drugs.

We have recently shown that the Topo IIα gene can be amplified in adenocarcinoma of the breast and a lung adenocarcinoma cell line (18,19). The Topo IIα locus is amplified in these tumors due to its position on chromosome 17q relative to ERBB2 (18). The growth factor receptor gene, ERBB2, is reported to be amplified in 10-30% of adenocarcinomas including breast and ovary (19,20,21,23,25,26,27). We therefore analysed ovarian tumors for co-amplification of ERBB2 and Topo IIα sequences. Of 86 tumors analysed for gene amplification, only one had ERBB2 amplification and the Topo IIα locus was not amplified in any sample. Thus, gene amplification does not regulate Topo IIα gene expression in these ovarian samples. The low frequency of ERBB2 amplification (1 out of 86), in the ovarian biopsies is contradictory to previously published frequencies of 20-32% (23,25,26,27). The marked difference in the frequency of ERBB2 amplification in the samples studied could be due to geographical differences in the populations studied as these may be exposed to different carcinogens. It is also possible that in some cases aneuploidy may be mistaken for low levels of amplification resulting in an over estimate of amplification.

In order to examine whether any other genetic changes have taken place at the Topo IIα locus, restriction fragment length polymorphisms (RFLP) analysis was employed (18,19). Using a RFLP for the Topo IIα locus, the frequency of heterozygosity at the Topo IIα locus in the tumor population was not significantly different from the normal population. These data suggest there to be very little genetic change

at the Topo IIα locus in ovarian cancer. However, estimates of heterozygosity in tumor populations in the absence of matched normal samples from each individual is a rather insensitive measure of genetic change.

In conclusion, we have shown that a commercially available antibody can be used to detect Topo IIα expression in 65% of ovarian tumors with a 16 fold range in expression. There were significantly higher levels of Topo IIα expression in the advanced stage and higher grade tumors. Analysis of the Topo IIα locus failed to reveal any gross genetic alterations which could account for the variation in levels of expression. These data confirm that Topo II is expressed in some ovarian cancers and is therefore a target for inhibition by Topo II poisons.

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Chapter 6

Glutathione transferase activity and isoenzyme composition in benign ovarian tumors, untreated malignant ovarian tumors, and malignant ovarian tumors after platinum / cyclophosphamide chemotherapy

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Abstract

Glutathione S-transferase (GST) isoenzyme composition, isoenzyme quantities and enzymatic activity were investigated in benign (n=4) ovarian tumors and malignant ovarian tumors, before (n=20) and after (n=16) chemotherapy. Enzymatic activity of GST in cytosols was measured by determining 1-chloro-2,4-dinitrobenzene conjugation with glutathione, cytosolic GST subunits were determined by wide pore reversed phase HPLC, using a S-hexylglutathione-agarose affinity column, and isoelectric focussing. Both GST activity and GST pi amount were not related to histopathologic type, differentiation grade, or tumor volume index in untreated malignant tumors. GST isoenzyme patterns were identical in benign tumors and malignant tumors before and after platinum / cyclophosphamide chemotherapy, while GST pi was the predominant transferase. Mean GST activity and GST pi amount were decreased ($p < 0.05$) in malignant ovarian tumors after platinum / cyclophosphamide chemotherapy compared to untreated ovarian malignant tumors. No relation was found in untreated ovarian tumors between GST pi amount and response to platinum / cyclophosphamide chemotherapy. Thus, within the limitations of the current study no arguments were found for a role for GST in in vivo drug resistance of malignant ovarian tumors to platinum / cyclophosphamide chemotherapy.

Introduction

Ovarian cancer is the fourth most frequent cause of cancer death in women and the gynaecological cancer with the highest mortality (Piver et al, 1991). The primary scheme for the treatment of patients with advanced ovarian carcinoma consists nowadays of cytoreductive surgery followed by systemic chemotherapy with platinum and cyclophosphamide containing combination regimens (Ozols & Young, 1991). Despite response rates of 50 - 80% to chemotherapy the above scheme cures only 15 to 20% of patients (Thigpen et al, 1989; Ozols & Young, 1991). These clinical data indicate, that in ovarian cancer intrinsic and acquired drug resistance occurs to platinum and cyclophosphamide containing regimens. In cell lines numerous mechanisms that can contribute to resistance to cisplatin have been identified, such as changes in membrane permeability, the ability to remove cytotoxic lesions from DNA, and changes in detoxification pathways (Andrews & Howell, 1990). With regard to cyclophosphamide and other anticancer drugs of the alkylating class, numerous studies point to the likelyhood that changes in GST isoenzyme composition and quantity contribute in an important way to the resistance of tumor cells (Waxman, 1990)

The aim of this study was to identify and quantify GST isoenzymes in benign and malignant tumors of the ovary, before and after chemotherapy and to relate the levels of these enzymes in untreated tumors to response of these tumors to chemotherapy. GSTs are a family of multifunctional cytosolic proteins that function as important enzymes of detoxification by catalyzing the conjugation of electrophilic compounds to glutathione, and the non-covalent binding of various lipophilic compounds (Boyer, 1989). In man, cytosolic GSTs have been divided into four major classes termed alpha (basic), mu (neutral), pi (acidic) and theta (Mannervik et al, 1985, Ogura et al, 1991). As these isoenzymes are known to have different substrate specificities, both the total GST activity and the isoenzyme composition may be important determinants of a tumors' ability to detoxify different chemotherapeutic agents (Mannervik & Danielson, 1988). In numerous human tumor cell lines resistant to cisplatin an enhanced GST content has been described, as well as in human tumor cell lines resistant to alkylating agents (for a review, see Meyer et al, 1990, Teicher et al, 1991, Ali-Osman et al, 1990, Ford et al, 1991). However, almost no data are available on GST activity and isoenzyme expression in tumors in vivo. In order to determine a possible role of GSTs in de novo and acquired resistance to platinum/cyclophosphamide chemotherapy we measured in this study the enzymatic activity, subunit composition and tissue concentration of GSTs in benign ovarian tumors and malignant ovarian tumors before and after chemotherapy. The relation between GST content of the untreated malignant tumors with histopathologic type, differentiation grade and tumor volume index, as well as the relation between the GST content of

the untreated malignant ovarian tumors with the clinical response to first line chemotherapy was investigated.

Materials and methods

Human materials

Tumor specimens were obtained from tumors operated at cooperating hospitals in the northern part of the Netherlands during the period 1989 - 1991. Tumor collection was supervised by a pathologist. After dissection samples were immediately frozen in liquid nitrogen and stored at -180°C until further analysis. In two patients, tumor specimens were obtained at first laparotomy and at second look operation after chemotherapy. In one untreated patient with ovarian cancer tumor specimens were obtained from the left and right ovarian tumor. In two patients four, and in two patients three specimens from different sites of the same tumor were obtained.

Pathological characteristics

The tumors were histologically classified according to the World Health Organisation classification using paraffin embedded tissue sections (Serov et al, 1973). One section per cm tumor diameter was made to get a good overall impression of the tumor histology. Carcinomas were graded into well-, moderately, and poorly differentiated ones (Sobre et al, 1982). Tumor volume index (percentage of malignant epithelial tissue in tumor specimen) was measured in the paraffin embedded sections. The tumor volume index was measured by a point counting technique, using a 42-point grid placed on a projection microscope at a magnification of 200-fold as described by Baak (Baak et al, 1988).

Classification of response to chemotherapy

Patients were defined as having a complete response (CR), when at second look operation no pathologic evidence of tumor was found, as having a partial response (PR), when at second look operation pathologic evidence of tumor was found, and tumor load was diminished (> 50%) in comparison to residual tumor after first operation, as having stable disease (SD), when at second look operation tumor load was comparable to residual tumor size after first operation, and as having progressive disease (PD), when during the course of chemotherapy at physical examination growing tumor masses were found.

HPLC separation and quantification of GST subunits and determination of GST activity

All actions were performed at 4°C, unless specified. All tumors aliquots (weights

ranging from 37-650 mg) were homogenized in 3.0 ml of Tris/HCl (25 mM, pH 7.4), using an ultra-turrax. To avoid contamination by connective tissue, the epithelium of the cystadenomas was dissected away from the cyst wall and used for further analysis. Cytosols were prepared by 90 min. centrifugation at 110,000 g. Cytosolic GST was purified as described previously (Bogaards et al, 1989) In brief a fixed amount of cytosol was applied to a 2 ml S-hexylglutathione-agarose affinity column, washed with 16 ml buffer containing 0.4 M NaCl, and eluted in the same buffer containing 5 mM of S- hexylglutathione. The eluates preceding the S-hexylglutathione alpha eluate were checked for GST activity, and usually contained less than 5% of the total applied enzymatic activity. The eluate was concentrated to approximately 0.2 ml, using a centricon PM 10 ultrafiltration tube (Amicon, Danvers USA), and 100 µl was applied to wide pore reversed phase HPLC (Vydac 105 TP 250 x 4.3 mm column). The subunits were eluted with a gradient of acetonitrile in water, both containing 0.1% trifluoroacetic acid (from 40 to 50% acetonitrile in 18 minutes, followed by a further increase to 53% in 5 minutes and isocrating elution for another 7 minutes). Detection was at 214 nm, while peak integration was performed using Nelson analytical software.

Concerning the method of quantification, it should be noted that possible theta class isoenzymes present in tumor tissues are not observed using this method, since they do not bind to the affinity matrix and show almost no enzymatic activity towards 1-chloro-2,4,dinitrobenzene (CDNB).

Human GST isoenzymes used for reference and quantification purposes was purified from a human placenta by means of S-hexylglutathione affinity chromatography and chromatofocusing, as described previously (van Ommen et al, 1990). For isoelectric focusing, a Pharmacia Phastsystem was used (pH-range 3-9 precoates). Enzymatic activity of GST (conjugation of CDNB with glutathione) was performed according to Habig (Habig et al, 1974).

Statistics

Statistical analysis of the distribution of tumor volume index, GST activity, and GST pi levels in the different groups was performed with the unpaired Student's t-test. Rank correlations were calculated by the method of Spearman. Only p-values < 0.05 were considered significant.

Results

Tumor histopathology, differentiation grade and tumor volume index

Tumor specimens from 40 patients were obtained. Four patients had benign cystadenomas, 20 patients untreated ovarian adenocarcinoma (2 patients FIGO stage I, 18

patients FIGO stage III). In 16 patients tumor specimens were obtained after Pt/Cy chemotherapy. For specification of chemotherapeutic regimens and response to these regimens in these patients, see table 1. Eight patients of the 16 patients had residual disease at second look laparotomy, performed within 1 month after the last course of chemotherapy, and eight patients had recurrent disease after a previous pathologically confirmed complete remission. Recurrence of disease in these eight patients occurred after a mean period of 19 months (range: 3-60 months). Treatment of these 16 patients with residual or recurrent disease after laparotomy consisted of varying second line chemotherapy regimens.

Table 1 Chemotherapy used in patients with residual or recurrent disease.

Patients	Chemotherapy	Response
1. res.d.	CP (6x)	PR
2. res.d.	CC (6x)	PR
3. res.d.	CC (6x)	PR
4. res.d.	CC (6x)	PD
5. res.d.	CP (6x)	PD
6. res.d.	CC (6x)	SD
7. res.d.	CAP (3x)	PR
8. res.d.	CC (5x)	PD
9. rec.d.	CC (6x)	CR
10. rec.d.	CC (9x)	CR
11. rec.d.	CC (6x)	CR
12. rec.d.	CC (6x), P/Vp (3x)	CR
13. rec.d.	CC (5x)	CR
14. rec.d.	CC (6x)	CR
15. rec.d.	CP (4x)	CR
16. rec.d.	CC (6x)	CR

Res.d., residual disease; rec.d., recurrent disease; CC, cyclophosphamide, carboplatin; CP, cyclophosphamide, cisplatin; CAP, cyclophosphamide, adriamycin, cisplatin; P/Vp, cisplatin i.v., etoposide i.p.; (nx), number of cycles.

Tumor histopathology, differentiation grade and tumor volume index.

For histopathologic type and differentiation grade of the untreated tumors, see Figures 1 and 2. No differences were found in mean tumor volume index in untreated ovarian cancer (52.3%, SD: 23.5) , residual disease (46.6%, SD: 28.3), and in recurrent disease (44.25%, SD: 18.5).

Enzymatic GST activity in cytosol

For mean GST activity in the different groups, see table 2. Although large interindividual variations were observed (range in adenomas: 0.07-0.20 U/mg cytosolic protein (cp), in untreated adenocarcinomas: 0.03-0.52 U/mg cp, and in adenocarcinomas after chemotherapy: 0.05-0.58 U/mg cp), the mean specific GST activity

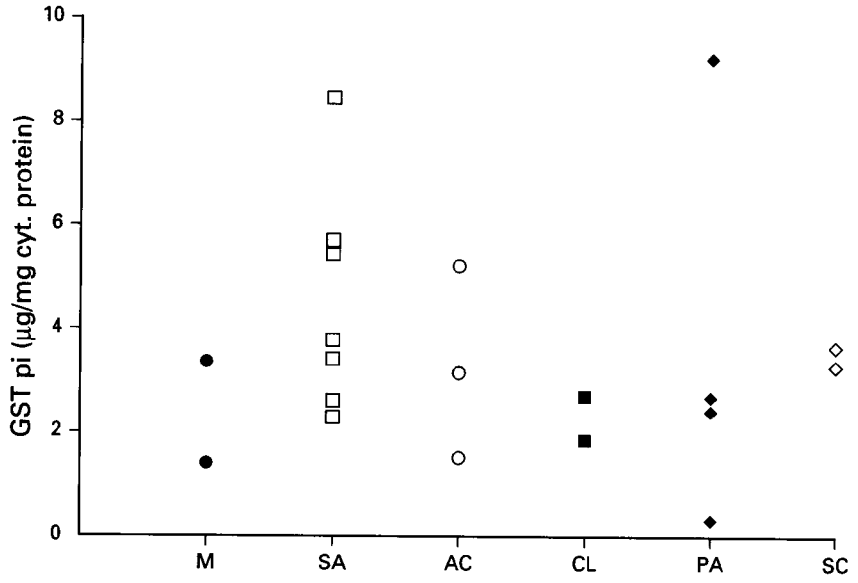

Figure 1 Amount of GST pi (µg/mg cytosolic protein) in different histopathologic types of untreated malignant ovarian tumors. M, mucinous adenocarcinoma; SA, serous adenocarcinoma; AC, adenocarcinoma; CL, clear cell adenocarcinoma; PA, papillary adenocarcinoma; SC, serous cystadenocarcinoma.

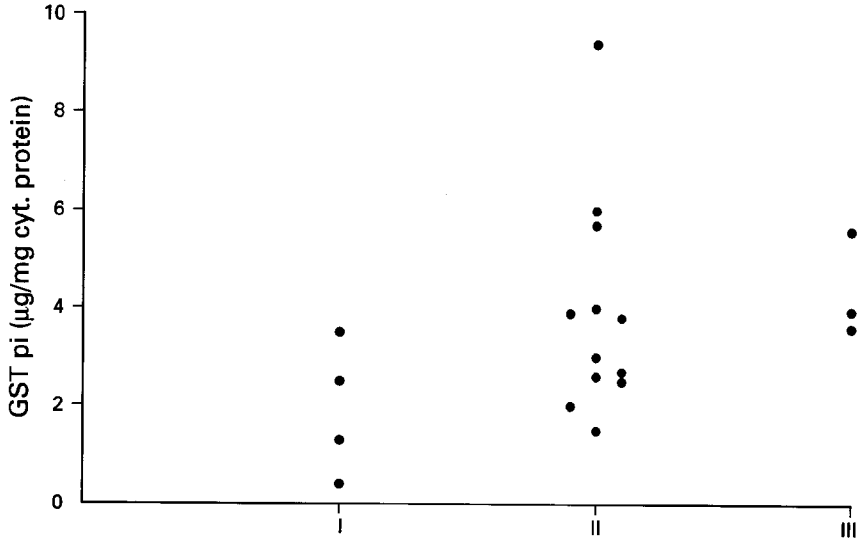

Figure 2 Amount of GST pi (µg/mg cytosolic protein) in relation to differentiation grade of untreated malignant ovarian tumors. Differentiation grade I, well differentiated; II, moderately differentiated; III, poorly differentiated.

towards conjugation of CDNB in tumors after chemotherapy was decreased ($p<0.05$) compared to untreated tumors. Expressed on the basis of the tumor weight, the differences were less pronounced. This is most likely due to a slight increase in the amount of cytosolic protein per gram tissue in tumors after chemotherapy (results not shown). Mean GST activity was increased ($p<0.05$) in untreated ovarian adenocarcinoma in comparison to benign tumors, but was equal in benign tumors and malignant tumors after chemotherapy. In the four patients from whom tumor specimens were obtained from respectively three and four different sites of the tumors the variation within these tumors was relatively small. The specific activity measured in cytosols in the different specimens always showed a standard deviation of less than 18%, both for untreated tumors, and tumors after chemotherapy. In one of the two patients from whom tumor specimens were obtained before and after chemotherapy the GST activity was higher after chemotherapy (0.16 vs. 0.58 U/mg cp), while in the other patient the GST activity was lower (0.52 vs. 0.18 U/mg cp).

Table 2 Tumor volume index, enzymatic GST activity and amount of GST pi in adenomas, untreated ovarian cancer, and ovarian cancer after chemotherapy.

Tumor	n	TVI	GST act.	GST pi
Benign	4		0.14 ± 0.05	1.50 ± 0.44
Untr.ca.	20	52.3 ± 23.5	0.26 ± 0.16*	3.61 ± 2.21*
Res./rec.	16	47.0 ± 23.3	0.18 ± 0.12**	1.78 ± 1.38**

TVI, mean tumor volume index ± SD; GST act., mean GST activity (CDNB conjugation) in U/mg cytosolic protein ± SD; GST pi, GST pi in µg/mg cytosolic protein ± SD; Untr.ca., untreated cancer; Res./rec., residual and recurrent disease; *, higher ($p< 0.05$) in comparison to benign tumors; **, lower ($p< 0.05$) in comparison to untreated malignant tumors.

Identification of GST subunits

Reversed phase HPLC separation of the affinity column eluate showed the subunit pi to be the predominant transferase present in all samples (Figure 3, lower panel). This was confirmed both by comparison with HPLC chromatograms of known mixtures of human liver GST (Figure 3, upper panel), comparison with purified GST pi isoenzymes and by co-elution of tumor transferases with purified GST pi. Isoelectric focusing of the purified tumor GST isoenzyme mixture, together with purified GST pi confirmed the acidic nature of the predominant tumor GST (results not shown). A minor subunit (< 3% of total cytosolic GST) was detected in most samples (Figure 3). Although not completely characterised, the corresponding isoenzyme has a molecular weight of ca. 27,000 dalton and a pI of 5.1. It possesses a rather low specific activity towards CDNB (ca. 5 U/mg). We have also observed this subunit in human liver and placenta (results not shown).

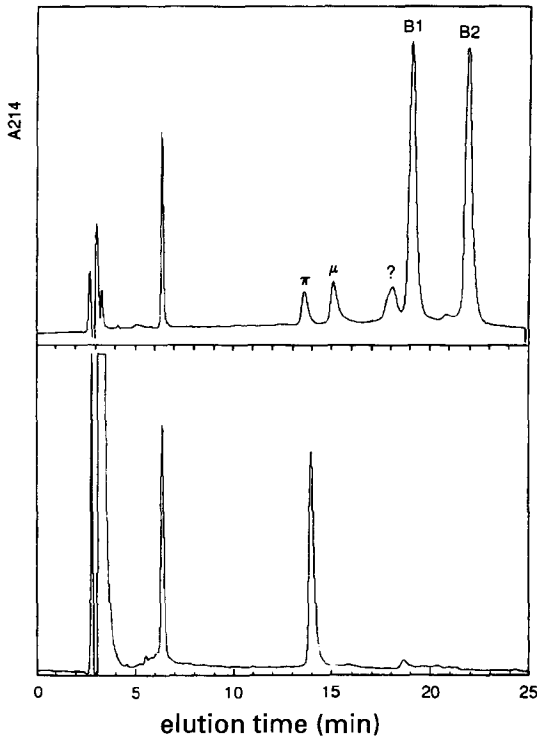

Figure 3 HPLC separation of glutathione S-transferase subunits, allowing for identification and quantification of the various subunits. Upper panel: Human GST subunits as present in liver. Lower panel: Elution profile of GST subunits purified from a tumor sample.

No differences in subunit composition were observed either within the benign and malignant tumors, or between the malignant tumors before and after chemotherapy. Isoelectric focusing of the purified tumor GST, together with markers, purified GST pi and other transferases confirmed the acidic nature of the tumor GST (results not shown).

Quantification of GST subunits

The mean amounts of GST pi in the various groups are shown in table 2. Similar to the effects observed with the cytosolic GST activity, the mean GST pi amount / mg cytosolic protein was lower ($p<0.05$) in the malignant tumors after chemotherapy as compared to the untreated tumors. When expressed relative to the tumor weight, the 50% decrease is not significant due to the large variation of GST pi amount in the individual tumors in the different groups (adenomas, range: 0.85 - 1.77 μg/mg cp; untreated adenocarcinomas, range: 1.40 - 9.21 μg/mg cp; adenocarcinomas after

chemotherapy, range: 0.45 - 5.68). The intratumor variation in the 4 patients from whom tumor specimens were obtained from respectively 3 and 4 different sites was relatively small. The amount of GST pi measured in cytosols in the different specimens always showed a standard deviation of less than 24%, both for untreated tumors, and tumors after chemotherapy. In the tumor specimens from both patients from whom tumor specimens were obtained before and after chemotherapy the amount of GST pi was lower after chemotherapy in comparison to before chemotherapy (4.51 vs. 9.21 μg GST pi/mg cp. and 1.85 vs. 2.86 μg GST pi/mg cp).

Relation activity / subunit composition

Figure 4 presents the relation between the cytosolic GST activity (CDNB conjugation) and the amount of GST pi in cytosol. A correlation coefficient of 0.83 was calculated for the total amount of samples. When separated into untreated tumors and tumors after chemotherapy, the correlation becomes less pronounced (0.79 and 0.70, respectively). The specific activity, as calculated from the slope of the regression line, is 51 nmol of CDNB conjugated per mg of GST pi (66 U/mg), as purified in our laboratory.

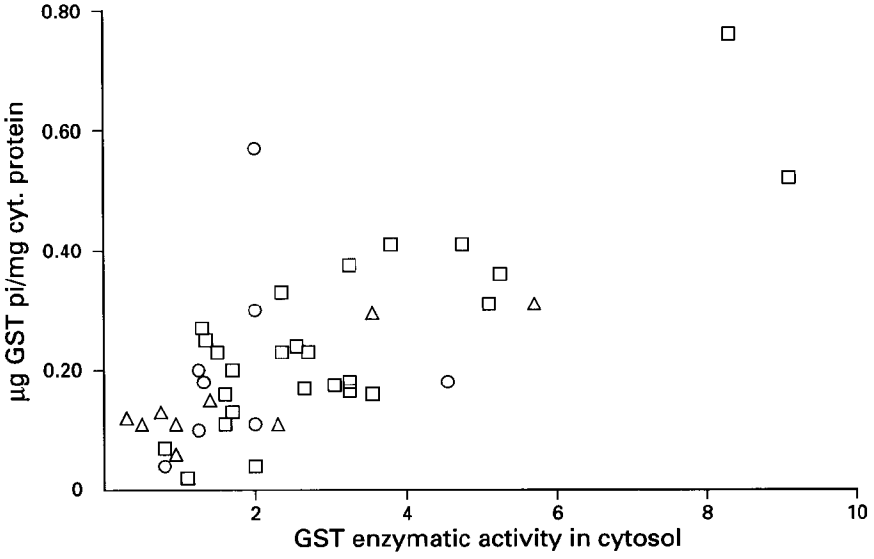

Figure 4 The relation between the enzymatic activity towards CDNB of cytosolic glutathione S-transferase and the amount of GST pi in the various tumor samples (■ = untreated cancers, △ = residual disease, ○ = recurrent disease).

Relations between GST pi amount / histopathologic type, differentiation grade and tumor volume index.

No relation was found between GST pi amount and histopathologic type, differentiation grade and tumor volume index of the untreated tumors (Figure 1, 2, and 5). Tumors after chemotherapy were not included to rule out possible influences of chemotherapy on GST pi amount.

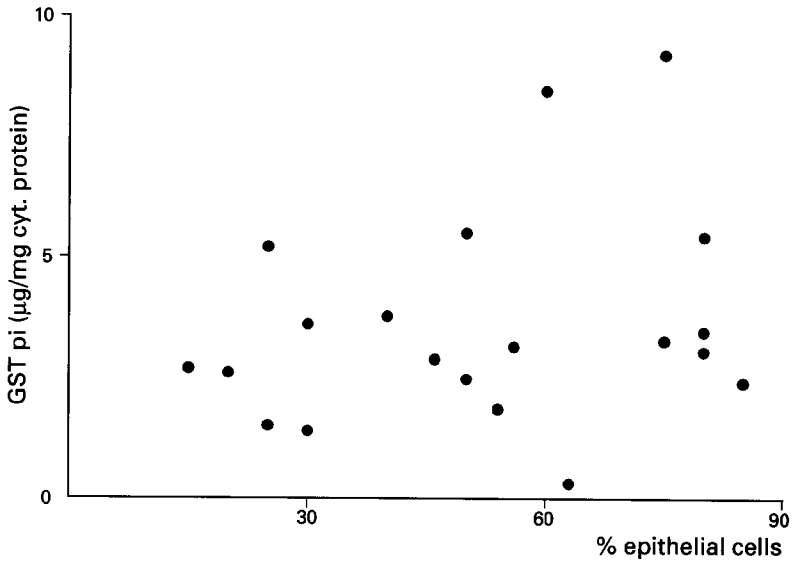

Figure 5 Amount of GST pi (µg/mg cytosolic protein) in relation to tumor volume index of untreated malignant ovarian tumors, r = 0.25, n.s.

GST pi amount and response to chemotherapy

Table 3 shows the amount of GST pi / mg cp., FIGO stage, chemotherapeutic regimen, and response to chemotherapy in 20 patients in which GST pi was measured in tumor specimens before treatment. Two of these patients did not receive chemotherapy because of a FIGO stage I, one patient was considered too old, and three patients received monotherapy because of high age. In two of the patients with PD during first line chemotherapy a second look laparotomy was performed to make another effort for debulking the tumor load.

Fig. 6 presents the relation between the GST pi amount / mg cp and response to platinum / cyclophosphamide chemotherapy. No rank correlation was found. In the group of eight patients with residual disease, from which tumors were obtained after

Table 3 Amount of GST pi, FIGO stage, chemotherapeutic regimen and response to chemotherapy.

Pts.	FIGO stage	Chemotherapy	GST pi	Response
1	IAI	-	1.40	NC
2	III	C/C (6x)	3.78	PR
3	III	-	1.51	NC
4	III	C/C (6x)	5.50	SD
5	III	C/C (6x)	5.21	CR
6	IAI	-	2.70	NC
7	III	C/P (6x)	5.43	CR
8	III	C/C (6x)	8.45	PR
9	III	C/C (5x)	9.21	PD
10	III	C/C (4x)	3.28	PD
11	III	C/C (5x)	2.61	PD
12	III	Cy (1x)	3.60	PD
13	III	CC (6x)	0.31	PR
14	III	Cy (4x)	2.39	PD
15	III	Cy (5x)	5.36	SD
16	III	CC (4x)	3.15	CR
17	III	CC (6x)	2.14	PR
18	III	CP (1x)	1.85	PD
19	III	CC (4x)	3.35	CR
20	III	CC (5x)	2.68	PD

Pts., patients; GST pi, μg/mg cytosolic protein; CC, cyclophosphamide, carboplatin; CP, cyclophosphamide, cisplatin; Cy, cyclophosphamide; CR, complete remission; PR, partial response; SD, stable disease; PD, progressive disease, NC, no chemotherapy.

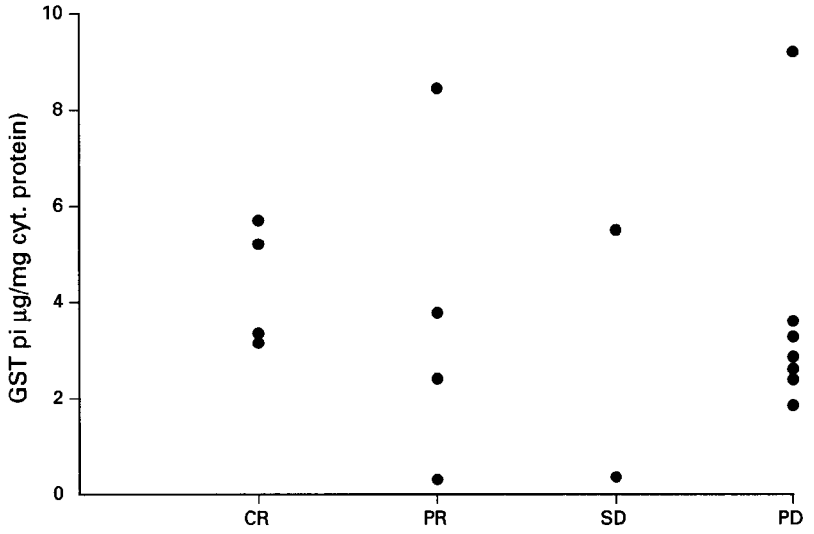

Figure 6 Relation between the quantity of glutathione S-transferase (amount of GST pi) and the response to platinum/cyclophosphamide treatment.

chemotherapy, progressive disease occurred in all patients despite second line and sometimes third line chemotherapy (various regimens). In the group of eight patients with recurrent disease after first line chemotherapy progressive disease occurred in five patients during second line chemotherapy (various regimens), one patient was lost for follow-up, and complete remission after second line chemotherapy occurred in two patients (one patient received six courses mitoxantrone, and one patient six courses of cisplatin/cyclophosphamide). Recurrence of disease after first line chemotherapy in these last two patients occurred after 4 and 5 years, respectively. In the patients with residual or recurrent disease no relation was found between GST pi levels in the tumor specimens and response to second line chemotherapy.

Discussion

Response to platinum / cyclophosphamide chemotherapy in ovarian cancer is quite variable. Well known prognostic factors in ovarian cancer are FIGO stage, differentiation grade, age of the patient, histopathologic type, residual tumor load after first laparotomy, morphometrical features and cellular DNA content (Baak et al, 1988). However, even within a group of patients with the same prognostic factors the response to chemotherapy is unpredictable. Therefore additional markers are needed to predict response to chemotherapy. Recently, a positive correlation between the enzymatic activity of GST and drug resistance to platinum and / or alkylating agents has been reported in cell lines (Lewis et al, 1988; Meyer et al, 1990, Teicher et al, 1991, Ali-Osman et al, 1990, Ford et al, 1991). Cell lines with in vitro acquired resistance or cell lines derived from resistant tumors showed higher GST levels than their non-resistant equivalents. Furthermore, expression of GST genes in cell lines led to a resistance against these types of compounds (Moscow et al, 1989). These observations suggest a role of GST in acquired drug resistance, possibly by means of an increased glutathione conjugation of antineoplastic agents or their reactive intermediates. Repeated treatment of patients with malignant ovarian tumors with platinum and / or alkylating agents may induce overexpression of GST. In this study both GST enzymatic activity and GST pi (which was the dominant GST isoenzyme) levels were lower in tumors obtained after treatment with platinum / cyclophosphamide in comparison to untreated tumors. However, as in most in vivo studies also our study is characterised by several complicating factors. Even in the patients with residual disease after chemotherapy the period between last course of chemotherapy and time of excision was at least four weeks, and therefore a possible transient rise in GST levels may be missed. Our series is small, and the range of histopathological types of tumors is wide. In only two patients tumor specimens were obtained before and after chemotherapy. GST pi levels were lower after chemotherapy in these two patients

with paired specimens, while in one patient GST activity was lower, and in the other GST activity was higher after chemotherapy.

Lower GST activity after chemotherapy is in agreement with the findings of Djuric et al, who found decreased activity of GST in malignant ovarian tumors after platinum / cyclophosphamide chemotherapy (Djuric et al, 1990). However, they did not measure isoenzyme patterns of GSTs, and did not relate GST level to response to chemotherapy.

In this study the acidic pi class GST was the most abundant GST form in benign ovarian tumors, and in malignant ovarian tumors, as was found by others in different human tumors, including lung, colon, bladder, and breast tumors (Di Ilio et al, 1985, 1988; Carmichael et al, 1988; Shea et al, 1988, 1990). Lewis et al (Lewis et al, 1989) described extremely high levels of the alpha class subunit in one malignant ovarian tumor, but this finding could not be confirmed by us in 36 malignant ovarian tumors. The isoenzyme composition in the residual and recurrent malignant ovarian tumors after platinum / cyclophosphamide chemotherapy did not change in comparison to the untreated malignant ovarian tumors. No other data exist in literature regarding GST isoenzyme patterns in malignant human tumors before and after chemotherapy. GST pi levels correlated well with GST activity in treated and untreated tumors (Figure 4). The one outlying point belongs to the patient with specimens obtained before and after chemotherapy, higher GST activity and lower GST pi level after chemotherapy. The isoenzyme pattern of the residual tumor of this patient showed no marked differences as compared to the corresponding primary tumor. Although one explanation for the disparity in GST activity and GST pi level may be a 'missed' isoenzyme in the HPLC assay, in our opinion this observation can not be explained by the presence of major amounts of other isoenzymes.

Mean GST pi level was higher ($p<0.05$) in untreated malignant ovarian tumors compared to benign tumors. This is in agreement with the findings for other human tumors, such as lung, colon, bladder, and breast tumors, where GST pi levels were higher in malignant tissue in comparison to the adjacent benign tissue (Shea et al, 1988; Moscow et al, 1989, Howie et al, 1990). However, mean GST pi level was equal in benign ovarian tumors and malignant ovarian tumors after platinum / cyclophosphamide chemotherapy.

Above mentioned in vitro studies suggested a relation in GST pi levels and resistance to chemotherapy. Uncertainty remains however, as to whether data describing mechanisms of resistance in vitro are relevant in human tumors. In earlier work we described no changes in P-glycoprotein and lower topoisomerase II in malignant ovarian tumors after platinum / cyclophosphamide chemotherapy in comparison to untreated tumors (van der Zee et al, 1991). In the current study no relation could be found between GST pi levels in untreated malignant ovarian tumors and response of these patients to platinum / cyclophosphamide chemotherapy. So far no further data

exist in literature on GST pi levels in tumors and response to platinum / cyclophosphamide chemotherapy. In patients with human breast tumors no relation was found between GST pi expression and in vitro chemosensitivity to doxorubicin (Keith et al, 1990), and in another study in human breast tumors no relation was found between GST pi content and other prognostic factors (Shea et al, 1990). Kim et al also did not find GST pi expression as an indicator of response to adriamycin in 15 human tumors (Kim et al, 1991). Lower levels of GST pi after platinum / cyclophosphamide chemotherapy and no relation of GST pi levels with response to platinum / cyclophosphamide chemotherapy both do not suggest an important role of GST pi in in vivo drug resistance. However, for the assays as used in our study homogenization of tissue is required. In this way subpopulations of tumor cells with high GST pi levels can be missed. Terrier et al found heterogeneity of expression of GST pi among different normal human tissues and also heterogeneity of GST pi expression within the same tissue using an immunohistochemical detection technique (Terrier et al, 1990). Recently, Rahilly et al also found heterogeneity of GST isoenzyme expression in benign and untreated malignant ovarian tumors (Rahilly et al, 1991). Perhaps the small subpopulations with high GST pi levels will eventually determine the response of the tumor to chemotherapy, and therefore, although not in favour for a significant role for GST pi as a marker of clinical drug resistance, our study does not rule out a possible role of GST isoenzymes in clinical drug resistance. In the future determination of GST pi levels with HPLC as well as an indirect immunohistochemical technique, using a polyclonal antibody against GST pi, can elucidate this problem. Another complicating factor in evaluating GST and glutathione levels in tumor specimens is the fact, that GSTs and glutathione are parts of a complicated detoxification system, of which glutathione and GSTs steady state are often used to determine the level of this detoxification system, as was done for GSTs in the current study. Insight in the kinetics of the glutathione and GST status should give a more dynamic representation of the continuous availability of this defence (Meijer et al, 1990b). However, our findings, that GST pi levels in untreated tumors showed no relation to response to chemotherapy, and that GST activity and GST pi levels were decreased after platinum/cyclophosphamide chemotherapy in comparison to untreated tumors, make the eventual use of inhibitors of GST enzymes, such as ethacrynic acid or piriprost as modulation strategy in enhancing sensitivity of malignant tumors to platinum / cyclophosphamide chemotherapy not very promising (Schilder et al, 1990).

Our study indicates that other in vitro well established mechanisms of drug resistance, such as decreased cell membrane transport of platinum, other detoxification pathways, and changes in repair of platinum DNA adducts, need to be validated in vivo, as was done for GSTs in the present study.

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Chapter 7

The value of P-glycoprotein, glutathione S-transferase pi, c-erbB-2 and p53 as prognostic factors in ovarian carcinomas

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Abstract

Purpose *To determine the prognostic value of immunostaining of P-glycoprotein (P-gp), glutathione S-transferase (GST) pi, c-erbB-2 and p53 in patients with advanced stage ovarian carcinoma with respect to response to chemotherapy and (progression free) survival ((PF)S).*

Patients and methods *Immunostaining of P-gp, GST pi, c-erbB-2, and p53 was performed on paraffin-embedded sections of 89 primary tumors and 38 residual tumors after chemotherapy (P-gp and GST pi) in a well documented series of patients with advanced ovarian carcinomas uniformly treated with platinum and adriamycin containing chemotherapy with long term follow-up. The results of immunostaining were related to clinico-pathologic prognostic factors in ovarian carcinomas and (PF)S.*

Results *P-gp and GST pi immunoreactivity were present in 13 cases (15%) and 79 cases (89%), respectively, and were not associated with any other clinico-pathologic prognostic factor or PFS. C-erbB-2 immunoreactivity was present in 18 cases (20%), and was associated with undifferentiated histiotype ($p<0.05$), but not with (PF)S. P53 immunoreactivity was present in the nuclei of 31 cases (35%) and cytoplasm of 9 cases (10%). Nuclear p53 staining was associated with grade III tumors, presence of > 1 liter ascites and residual tumor after first laparotomy > 2 cm. The following factors were associated with shorter PFS in log rank analysis: stage III or IV disease (relative risk (RR): 5.9, 95% confidence interval (CI): 2.3-14.7), no response to chemotherapy (RR: 5.2, 95% CI: 3.0-8.8), nuclear p53 staining (RR: 3.3, 95% CI: 2.0-5.6), presence of > 1 liter ascites (RR: 3.2, 95% CI: 1.9-5.3), residual tumor after*

first laparotomy > 2 cm (RR: 3.0, 95% CI: 1.8-5.2), and age > 50 years (RR: 1.9, 95% CI: 1.1-3.2). Nuclear p53 staining was also associated with shorter overall survival (RR: 2.6, 95% CI: 1.7-3.8). After adjustment for presence of > 1 liter ascites, and age > 50 years nuclear p53 staining did not retain independent prognostic significance in stage III/IV tumors. The frequency of P-gp staining in patients with residual tumors after chemotherapy (18/38 cases) was higher in comparison to untreated tumors (13/89 cases) (p<0.001). No combination of immunohistochemical or clinicopathologic parameters was able to predict response to chemotherapy adequately.

Conclusions *Nuclear immunoreactivity of p53 in ovarian carcinomas is associated with shorter (PF)S and determinants of more aggressive tumor growth. Higher frequency of P-gp immunoreactivity in residual tumors after chemotherapy points to induction of P-gp in ovarian carcinomas by platinum and adriamycin containing combination chemotherapy. The determination of P-gp, GST pi, c-erbB-2 and p53 does not permit more adequate prediction of response to chemotherapy.*

Introduction

Epithelial ovarian carcinomas are responsible for the death of more women than all other gynecologic malignancies combined in the Western world. Ovarian carcinomas are detected in an advanced stage of the disease in about 80% of patients. Standard treatment of patients with advanced ovarian carcinoma consists of surgical debulking in combination with platinum containing combination chemotherapy. Well known adverse prognostic factors include advanced stage of disease, presence of bulky residual disease after initial surgery, older age, performance status, high tumor grade, and presence of clear cell or mucinous adenocarcinoma. More recently, tumor DNA ploidy, and pre- and postoperative serum CA 125 levels have been proposed as independent prognostic factors in ovarian carcinomas.[1] Combinations of the current available prognostic factors can adequately predict the long-term survival of patients before initiation of chemotherapy. However, prediction of response to chemotherapy in individual patients is not possible.[2] New prognostic factors related to response to chemotherapy and/or tumor cell growth could have additive value in this respect.

Mechanisms of drug resistance and their reversal are among the most important issues in the treatment of patients with ovarian carcinoma. Possible mechanisms are changes in drug transport by overexpression of drug extruders in the cell membrane such as P-glycoprotein (P-gp), enhanced detoxification of drugs by cytosolic sulfhydryl molecules (glutathione, glutathione S-transferase (GST)), changes in the targets of drugs (topoisomerases), and enhanced repair of DNA damage.[3] Differences in expression of tumor suppressor genes and/or oncogenes may not only have implications for tumor aggressiveness, but also influence the sensitivity of tumors to drugs.[4,5]

P-gp is an energy-driven, M_r 170,000 membrane efflux transporter. Its expression has been linked to the presence or development of multidrug resistance. By preventing their cellular accumulation P-gp plays a role in resistance to natural drugs such as vinblastine, colchicine, adriamycin, and taxol.[6,7]

GSTs are a family of multifunctional detoxification enzymes. Enhanced GST content has been described in tumor cell lines resistant to cisplatin, alkylating agents, and/or adriamycin.[8] Recently, we investigated GST isoenzyme patterns in ovarian carcinomas. It appeared that GST pi is the predominant transferase in these tumors.[9]

The c-erbB-2 oncogene (also known as HER2/neu oncogene), located on chromosome 17q21, encodes for a M_r 185,000 transmembrane phosphoglycoprotein with intrinsic tyrosine kinase activity. The c-erbB-2 oncogene is closely related to the epidermal growth factor receptor.[10] Amplification or overexpression of the c-erbB-2 oncogene may lead to increased overall tyrosine kinase activity and, thus, more aggressive tumor growth.[11] C-erbB-2 overexpression may also influence sensitivity to cytotoxic drugs such as cisplatin and adriamycin.[5]

Changes in the p53 tumor suppressor gene are among the most common genetic alterations found in human malignancies.[12] The wild type (wt) nuclear phosphoprotein p53 binds to specific DNA sites and stimulates the expression of downstream genes that negatively control growth and/or invasion.[13] Loss of wt p53 by different pathways (such as mutations) may lead to more aggressive tumor growth. Loss of wt p53 function by mutations in the p53 gene has shown to be a negative prognostic factor in different malignancies.[14] In contrast, it has been suggested, that, in particular in stressed cells, wt p53 has an important role in cellular growth control. Increased wt p53 after X-ray or drug-induced DNA damage leads to cell cycle arrest, and thereby facilitates DNA damage repair. In vitro studies show that cells with loss of wt p53 function are more sensitive to DNA-damaging agents.[4] Theoretically, the presence of p53 mutations could be a possible positive prognostic factor in malignant tumors as a result of increased sensitivity to chemotherapy of malignant tumors after loss of wt p53.

In this study immunostaining of P-gp, GST pi, c-erbB-2, and p53 was related to conventional prognostic parameters in ovarian carcinomas as well as with PFS and response to chemotherapy in a well documented series of patients with advanced ovarian carcinoma with long term follow-up. Possible induction of expression of P-gp and GST pi by combination chemotherapy was investigated by immunostaining of residual tumors at second look laparotomy.

Materials and methods

Patients

Between 1 April 1980 and 1 June 1987, 93 consecutive patients with advanced

ovarian carcinoma were treated and evaluated at the University Hospital Groningen. Final evaluation date was 1 May 1993. In four patients no paraffin-embedded blocks were currently available. All patients were initially treated with optimal tumor reductive surgery, and were staged according to the International Federation of Obstetrics and Gynecology (FIGO) classification.[15] Fifty-five patients had their first laparotomy in the referring hospital. Operative assistance was provided by a gynecologic oncologist from the University Hospital Groningen. Residual tumor after first laparotomy was categorized as follows: residual tumor smaller than 2 cm and residual tumor larger than 2 cm in diameter. The eligibility criteria for chemotherapy were: patients with ovarian carcinoma, FIGO stage IC, IIC, III and IV, under 70 years of age. Patients with a WHO performance score of 3 or 4, creatinine clearance below 90 ml/min, bilirubin over 30 μmol/l, overt cardiac failure, infectious foci, or a second malignancy were excluded. Combination chemotherapy was initiated within a median of three weeks (range 2-6 weeks) after laparotomy. Seventy-six patients were treated with a combination of cyclophosphamide 500 mg/m^2 d1 i.v., adriamycin 35 mg/m^2 d1 i.v., and cisplatin 20 mg/m^2/d d1-d5 i.v. (CAP-5) and 13 patients with a combination of adriamycin 35 mg/m^2 d1 i.v., cisplatin 20 mg/m^2/d d1-5 i.v., cyclophosphamide 100 mg/m^2/d d15-d28 p.o., and hexamethylmelamine 100 mg/m^2/d d15-28 p.o., (CHAP-5). Patients received six (CAP-5) respectively five (CHAP-5) cycles of combination chemotherapy unless after three cycles no response to chemotherapy was observed by physical examination or at second look laparotomy. Evaluation relaparotomy was planned after the first three cycles of chemotherapy only in patients with a clinical response. After three cycles of chemotherapy tumor response was classified according to WHO criteria in 70 patients with evaluable disease after first laparotomy (excluding 10 stage IC, three stage IIC, and eight stage III patients with complete debulking at first laparotomy).[16] After chemotherapy and surgery, all patients were followed up to 10 years with gradually increasing intervals.

Histology

Primary tumors were classified according to the WHO classification using paraffin embedded tissue.[17] Carcinomas were graded into well (grade I), moderately (grade II), and poorly (grade III) differentiated ones, as described by Sobre et al.[18]

Antibodies

The JSB-1, GST pi, CB11, and CM-1 antibodies were purchased from Novocastra Laboratories Ltd, Newcastle upon Tyne, UK. The monoclonal antibody JSB-1 (dilution 1/20) is specific for a conserved epitope on the plasma membrane P-glycoprotein. GST pi (dilution 1/50) is a rabbit polyclonal antibody raised against purified protein from the cytosol of human chronic lymphocytic leukaemic spleen. CB11 (dilution 1/25) is a monoclonal antibody recognizing the internal domain of the c-

erbB-2 oncoprotein. The polyclonal antibody CM-1 (dilution 1/200) recognizes both wt and mutant p53 protein in formalin-fixed, paraffin-embedded archival material.

Immunohistochemistry

Five μm formalin- or occasionally Bouin-fixed, paraffin-embedded sections were cleared in xylene and rehydrated in graded alcohols. With JSB-1 and CM-1 antibodies microwavé antigen retrieval using target unmasking fluid (TUF) was performed with modifications, as described by van den Berg et al.[19] In brief, sections were submerged in a Coplin jar filled with TUF, diluted 1:5 in distilled water. The Coplin jar was placed for 18 min (6 cycles of 3 min) in a microwave oven set at 450 W. The jar was allowed to cool down to 40-45°C. Subsequently, the sections were rinsed three times in phosphate buffered saline, pH 7.4. After incubation with the primary antibodies for 60 min at 37°C, slides were exposed to the secondary biotinylated rabbit anti-mouse IgG for monoclonal antibodies and to the secondary biotinylated swine anti-rabbit IgG for the polyclonal antibodies, followed by the amplification system streptavidin-biotin complex. The slides were then treated with diaminobenzidine and hydrogen peroxide for 10 min and counterstained with hematoxylin. A control slide in which normal mouse or rabbit serum was substituted for the primary antibody served as a negative control. Positive controls were: adrenal cortex for P-gp, a breast carcinoma with well established p53 immunostaining for p53, human liver for GST pi, and a breast carcinoma with well established c-erbB-2 immunostaining for c-erbB-2 detection.

All sections were read independently by two pathologists (HH and AJHS) without knowledge of the clinical data. Samples were scored as negative when there was complete absence of staining, or only scattered positive cells were found. Samples were scored as positive when > 5% of tumor cells had plasma membrane and/or cytoplasmatic staining (P-gp, c-erbB-2) or cytoplasmatic and/or nuclear staining (GST pi, p53). The evaluations of the two observers were in concordance in 95% of cases. Discordant data were discussed at the microscope, until consensus was reached.

Statistical methods

Differences in (PF)S from the first laparotomy were analyzed using log-rank statistics.[20] The influence on (PF)S of the different clinical, histopathologic, and immunohistochemical data as categorical variables was determined by trend analysis and corrected for trends when appropriate. The results were expressed as relative risks (RR) of death or progression of disease. Associations between the different parameters were determined using chi-square analysis. Only p- values < 0.05 were considered significant.

Results

Patients

The first two columns of table 1 summarize the clinico-pathologic data of our series. No patients were lost for follow-up. After chemotherapy 32 (35%) patients had no progression of disease, whereas 57 (65%) had progressive disease.

The median PFS was 26 months, with a range of 0-144+ months. Thirty-four (38%) patients are alive with no evidence of disease. The median overall survival was 46 months, with a range of 3-144+ months. In 70 patients a second look laparotomy was performed; 29 patients had achieved a complete remission, while 41 patients had residual tumor after three courses of chemotherapy.

Table 1 Distribution of P-gp, GST pi, c-erbB-2, and p53 immunostaining according to clinico-pathologic parameters in 89 advanced ovarian carcinomas

	No. of patients	P-gp + no. (%)	GST pi + no. (%)	c-erbB-2 + no. (%)	p53 +^{n} no. (%)	p53 +^{c} no. (%)
All cases	89	13 (15)	79 (89)	18 (20)	31 (35)	9 (10)
Age						
≤ 50	38	5 (13)	34 (89)	7 (18)	10 (26)	2 (5)
> 50	51	8 (16)	45 (88)	11 (22)	21 (41)	7 (14)
FIGO stage						
I/II	20	3 (15)	19 (99)	6 (30)	4 (20)	3 (15)
III/IV	69	10 (14)	60 (87)	12 (17)	27 (40)	6 (9)
Tumor grade						
I	26	4 (15)	22 (85)	4 (15)	6 (23)	4 (15)
II	35	6 (17)	33 (95)	4 (11)	10 (29)	3 (9)
III	28	3 (10)	24 (86)	10 (36)	15 (54)	2 (7)
Tumor type						
serous	54	7 (13)	48 (89)	10 (19)	21 (39)	5 (9)
mucinous	9	2 (22)	9 (100)	1 (11)	2 (22)	2 (22)
endometroid	7	1 (14)	5 (71)	1 (11)	0	0
clear cell	10	2 (20)	10 (100)	1 (10)	2 (20)	1 (10)
undifferentiated	9	1 (11)	7 (78)	5 (56)	5 (56)	1 (10)
Ascites						
< 1 liter	63	9 (14)	59 (94)	12 (19)	16 (25)	7 (11)
> 1 liter	26	4 (15)	20 (77)	6 (23)	15 (58)	2 (7)
Residual tumor after first laparotomy						
< 2 cm	50	9 (18)	42 (84)	9 (18)	10 (20)	6 (12)
> 2 cm	39	4 (10)	37 (95)	9 (23)	21 (54)	3 (8)

p53^{n}: nuclear immunostaining; p53^{c}: cytoplasmatic immunostaining. Percentages (%) are calculated as percentages of number of patients in the second column.

Immunohistochemistry

Table 1 summarizes P-gp, GST pi, c-erbB-2 and p53 staining results in relation to clinico-pathologic parameters. In the adrenal gland (control) both membraneous and cytoplasmatic staining for P-gp was seen. In ovarian carcinomas three staining patterns were encountered; in 16/89 cases (18%) a granular (Golgi) staining pattern was seen, and in 13/89 cases (15%) a diffuse cytoplasmatic (9 cases) or predominantly membraneous (4 cases) staining pattern. Finstad et al have reported that the granular staining pattern may be due to contaminating antibodies to blood group A antigen in some of the JSB-1 monoclonal antibodies lots.[21] All 16 patients with a granular staining pattern had blood group A, and therefore this staining pattern was regarded as nonspecific and not included in the statistical analysis. Only three out of the 13 patients (23%) with a diffuse cytoplasmatic or membraneous staining pattern had blood group A (frequency of blood group A in Western population: 35%). In these 13 patients no granular staining pattern was found. Diffuse cytoplasmatic and membraneous P-gp immunostaining were not related to any other clinicopathologic prognostic factor.

Nuclear and/or cytoplasmatic staining of GST pi was found in 79/89 cases (89%). All 79 cases had abundant and homogeneous GST pi staining. In 54 cases GST pi staining was cytoplasmatic, in 21 cases nuclear, and in four cases both cytoplasmatic and nuclear. Because these distinctive patterns were not associated with any prognostic parameter or (PF)S (results not shown), all cases with GST pi staining were regarded as one group. In 10 cases no GST pi staining was found. GST pi positivity (regarded as one group) was not associated with any other prognostic factor.

Both cytoplasmatic and membrane-bound c-erbB-2 staining was found in 18/89 cases (20%). In sections comprising normal ovarian tissue no staining was found in the surface epithelium, which confirms the specificity of our detection method. C-erbB-2 positivity was associated ($p<0.03$) with undifferentiated carcinomas (5/9 cases; 55%), but not with any other prognostic factor.

Nuclear staining of p53 was found in 31/89 cases (35%) (fig. 1), and cytoplasmatic staining in 9/89 cases (10%). Staining patterns were homogeneous for both nuclear and cytoplasmatic p53 staining. Nuclear p53 staining was associated with grade III ovarian carcinomas ($p<0.05$), the presence of > 1 liter ascites ($p<0.05$) and with residual tumor > 2 cm. after first laparotomy ($p<0.05$), but not with any other prognostic factor.

Since platinum and/or adriamycin containing chemotherapy may induce expression of P-gp and GST pi, these markers of drug resistance were also evaluated in patients with residual tumor after chemotherapy (n=41). In three cases the amount of residual tumor was very small and these cases were not suitable for immunostaining. Table 2 summarizes the results of P-gp and GST pi immunostaining in patients with tumor samples obtained before and after chemotherapy. Cytoplasmatic P-gp staining after

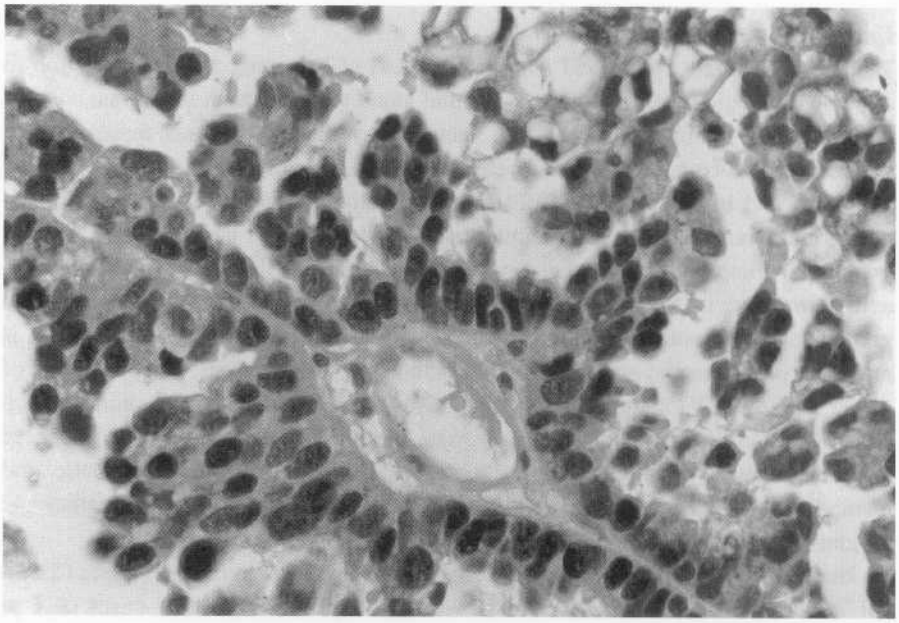

Figure 1 Papillary serous adenocarcinoma. High index of p53 staining in nearly all tumor cell nuclei.

chemotherapy was found in 18/38 cases (48%), which is more frequent than in untreated patients (15%) ($p<0.001$). The staining pattern was diffuse cytoplasmatic, and the intensity of staining was comparable to the untreated positive tumors. Fourteen patients had positive P-gp staining in the residual tumor, while the primary tumor was negative. GST pi staining in residual tumors was found in 35/38 cases (92%), which is comparable to GST pi staining in primary tumors (79/89 cases (89%)). GST pi staining was cytoplasmatic in 31 cases, nuclear in two cases, and both cytoplasmatic and nuclear in two cases. The intensity of GST pi staining in residual tumors was comparable to GST pi staining in primary tumors.

Survival analysis

Table 3 summarizes the results of log rank analysis of the prognostic impact on progression free survival of clinical, histopathologic and immunohistochemical parameters for all patients and stage III/IV patients. As PFS is a more adequate reflection of efficacy of first line chemotherapy than overall survival the data on PFS are presented. Significant prognostic factors in all stages for a shorter PFS were: stage III/IV (RR: 5.9, 95% Confidence Interval (CI):2.3-14.7), no response to or stable disease during chemotherapy (RR: 3.7, 95% CI:2.1-6.4), nuclear p53 immunostaining (RR: 3.3, 95% CI:2.0-5.6), presence of > 1 liter ascites (RR: 3.2, 95% CI:1.9-5.3),

Table 2 Influence of chemotherapy on Pgp and GST pi immunostaining in 38 patients with tumor specimens obtained before (B) and after (A) chemotherapy.

P-gp staining:	No. of patients
B-/A-:	19
B-/A+:	14
B+/A-:	1
B+/A+:	4

GST pi staining:	No. of patients
Bc/Ac:	23
Bn/Ac:	8
Bn/An:	2
Bnc/A-:	2
Bc/Anc:	1
Bc/A-:	1
Bn/A-:	1

B: before chemotherapy; A: after chemotherapy; -: negative; +: positive; c: cytoplasmatic staining; n: nuclear staining; nc: nuclear and cytoplasmatic staining.

residual tumor > 2 cm after first laparotomy (RR: 3.0, 95% CI:1.8-5.2), and age > 50 years (RR: 1.9, 95% CI:1.9-3.2). Significant prognostic factors in stage III and IV for a shorter PFS were no response to or stable disease during chemotherapy (RR: 3.5, 95% CI:2.0-6.0), nuclear p53 immunostaining (RR: 2.8, 95% CI:1.3-4.2), age > 50 years (RR: 2.7, 95% CI:1.2-4.1), presence of > 1 liter ascites (RR: 2.2, 95% CI:1.3-3.7), and residual tumor > 2 cm after first laparotomy (RR: 2.1, 95% CI:1.2-3.7). Tumor type and tumor grade, c-erbB-2, P-gp, cytoplasmatic p53 and GST pi immunostaining had no prognostic impact. Despite the fact that a variety of second line chemotherapy regimens were given after progression of disease, the figures for p53 immunostaining as calculated for overall survival were comparable to PFS (all stages, RR: 2.6, 95% CI:1.5-4.3); stage III/IV, RR: 2.0, 95% CI:1.2-3.5). Figure 2 and figure 3 show the Kaplan-Meier curves for PFS and overall survival respectively for patients with and without nuclear p53 immunostaining. Patients with nuclear p53 staining had a shorter PFS in comparison to patients without p53 staining in all stages ($p<0.01$) and in stage III/IV ($p<0.05$). However, after adjustment for the presence of > 1 liter ascites (RR: 1.7, 95% CI:1.0-2.9) or age > 50 years (RR:1.5, 95% CI:0.9-2.7) this difference did not retain its independence in stage III/IV ovarian carcinomas, which was also found for overall survival.

Prediction of response to chemotherapy

No association was found between any immunohistochemical parameter and response to chemotherapy, as determined at second look laparotomy after three cycles of

Table 3 Log rank analysis for PFS of clinical, histopathologic, and immunohistochemical parameters in all stages, and stage III/IV.

Prognostic factor	RR (95% CI) All stages	RR (95% CI) Stage III/IV
Stage		
III/IV vs. I/II	5.9 (2.3-14.7)[d]	
Response to chemotherapy		
SD/PD vs. CR/PR	3.7 (2.1-6.4) [f]	3.5 (2.0-6.0) [f]
p53		
pos.[n] vs. neg.	3.3 (2.0-5.6) [f]	2.8 (1.3-4.2) [d]
pos.[c] vs. neg.	1.0 (0.4-2.8) ns	0.9 (0.7-1.8) ns
Ascites		
> 1 liter vs. < 1 liter	3.2 (1.9-5.3) [f]	2.2 (1.3-3.7) [c]
Residual tumor after first laparotomy		
> 2 cm vs. < 2 cm	3.0 (1.8-5.2) [f]	2.1 (1.2-3.7) [a]
Age		
> 50 vs. < 50 years	1.9 (1.1-3.2) [a]	2.7 (1.5-4.8) [d]
Tumor grade		
III vs. II/I	1.3 (0.8-2.3) ns	1.3 (0.7-2.2) ns
Tumor type		
MA/CI.C vs. others	1.0 (0.5-1.9) ns	1.0 (0.5-1.9) ns
GST pi		
pos. vs. neg.	0.9 (0.5-1.7) ns	0.8 (0.4-1.9) ns
c-erbB-2		
pos. vs. neg.	0.8 (0.4-1.5) ns	0.9 (0.5-1.6) ns
P-gp		
pos. vs. neg.	0.8 (0.6-1.2) ns	0.8 (0.5-1.3) ns

p53[n]: nuclear immunostaining; p53[c]: cytoplasmatic immunostaining; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; MA/CI.C: mucinous and clear cell adenocarcinoma; [a]: $p<0.05$, [b]: $p<0.03$, [c]: $p<0.01$, [d]: $p<0.003$, [e]: $p<0.001$, [f]: $p<0.0001$

chemotherapy in 70 patients with evaluable disease after first laparotomy (see table 4). No combination of clinicopathologic and/or immunohistochemical parameters adequately predicts the non-responding tumor phenotype. This is illustrated by the fact that response to chemotherapy was found in five out of eight patients with stage III/IV disease, age > 50 years, positive nuclear p53 staining, > 1 liter ascites, and residual tumor > 2 cm after first laparotomy.

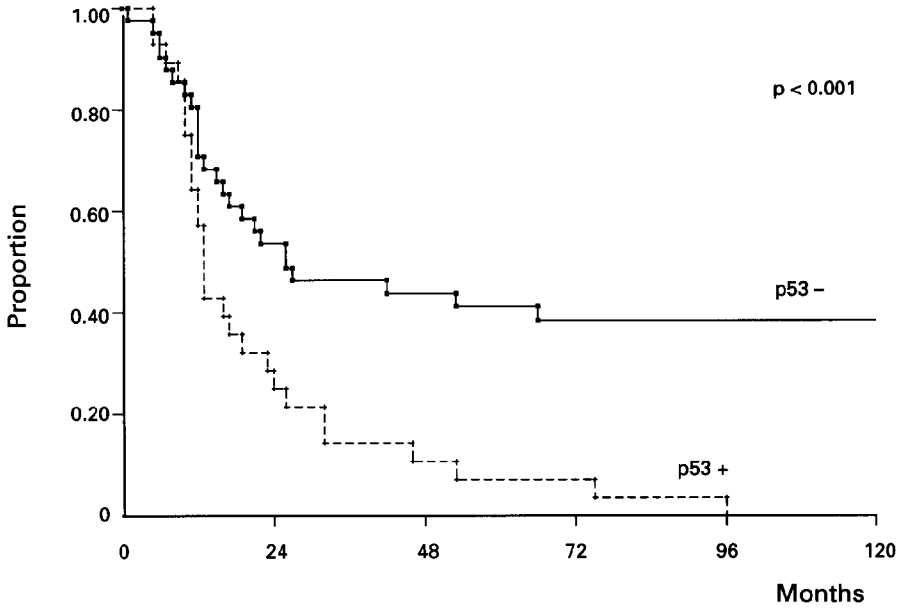

Figure 2a PFS for all stages by p53 staining characteristics.

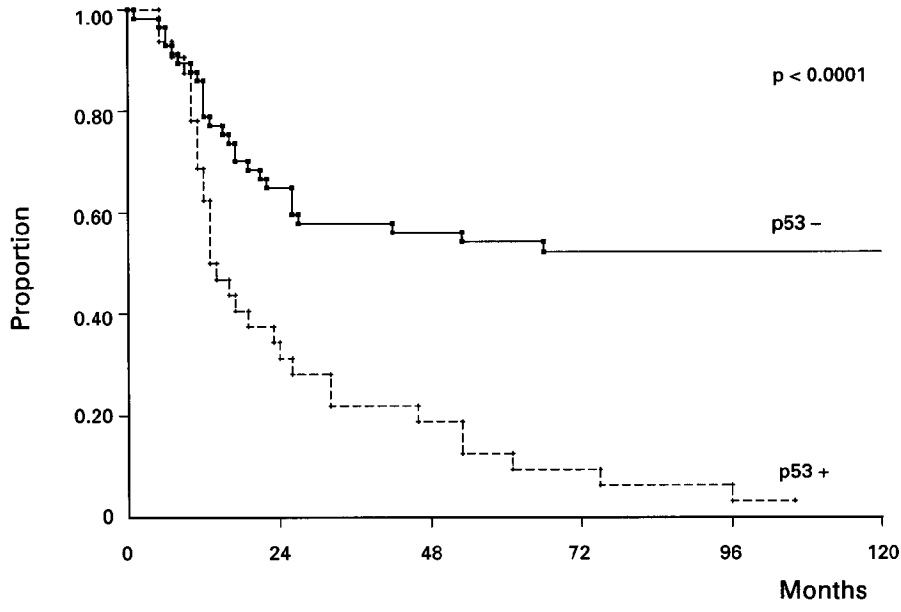

Figure 2b PFS for stage III/IV by p53 staining characteristics.

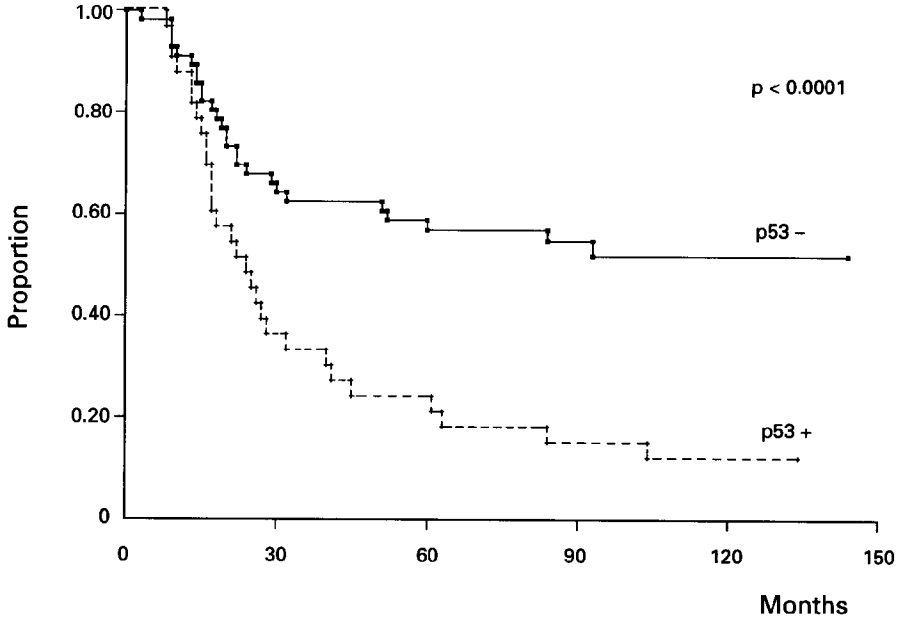

Figure 3a Overall survival for all stages by p53 staining characteristics.

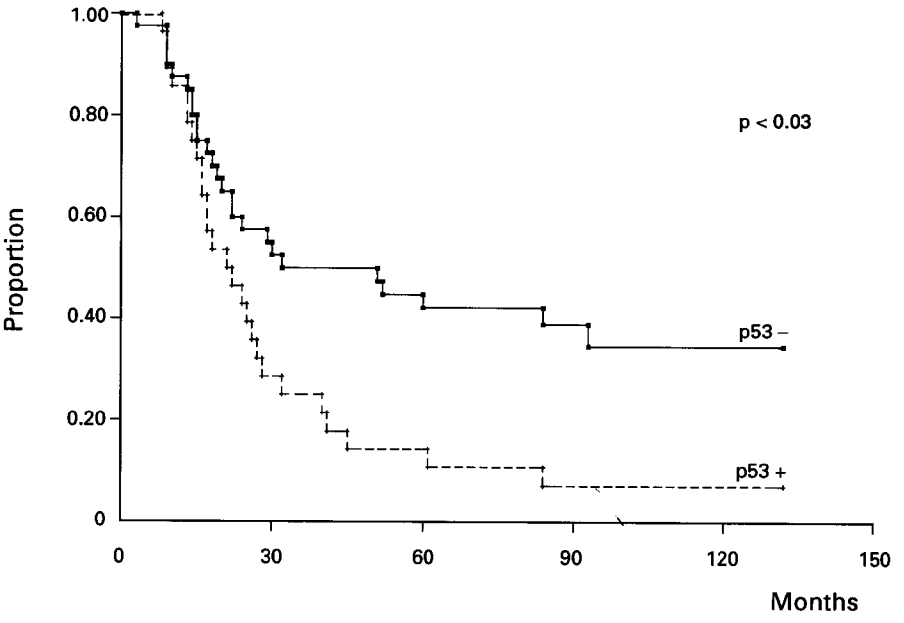

Figure 3b Overall survival for stage III/IV by p53 staining characteristics.

Table 4 Association between immunohistochemical parameters at diagnosis and response to chemotherapy in patients with evaluable disease after first laparotomy.

	Staining	Total no. pts.	CR	PR	SD	PD	p- value
P-gp	-	61	19	21	13	8	n.s.
	+	9	2	5	2	0	
GST pi	-	9	4	2	2	1	n.s.
	+	61	16	24	13	7	
C-erbB-2	-	55	16	20	12	7	n.s.
	+	15	5	6	3	1	
$p53^n$	-	38	16	11	6	5	n.s.
	+	32	5	15	9	3	

CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease; $p53^n$: nuclear immunostaining p53.

Discussion

In this immunohistochemical study performed on paraffin-embedded tissue, we found P-gp staining, using microwave antigen retrieval, in 13/89 (15%) untreated ovarian carcinomas. Conflicting data on the frequency of P-gp expression in ovarian carcinomas are obviously due to the different sensitivity of the detection techniques (Western/Northern blot, immunohistochemistry, RT-PCR).[22-24] In our present study the P-gp staining patterns were granular (Golgi), diffuse cytoplasmatic, and predominantly membraneous. These patterns of P-gp immunoreactivity are also encountered in the literature: (aspecific) Golgi membrane immunoreactivity has been linked to the presence of blood group antigen A[21], which was confirmed in our study. Diffuse cytoplasmatic immunostaining has been described in different malignancies such as malignant mesothelioma[25], while membraneous immunostaining especially has been described in P-gp expressing cell lines.[26] In our present and previous study P-gp staining was largely cytoplasmatic. It may be that this diffuse cytoplasmatic P-gp immunoreactivity is not specific for P-gp expression. However, in cell lines lower degrees of drug resistance were associated with cytoplasmatic staining, while higher degrees of resistance appeared to be associated with membraneous staining.[27] In the present study diffuse cytoplasmatic P-gp immunostaining was more frequently found in residual tumors after 3 cycles of adriamycin containing chemotherapy, and in fourteen patients diffuse cytoplasmatic P-gp immunostaining was present in residual tumor after (adriamycin) chemotherapy, while the primary tumor from the same patient did not show P-gp positivity. The induction of P-gp staining in residual tumors

suggests that diffuse cytoplasmatic P-gp staining may be related to P-gp expression, and that P-gp may have a role in drug resistance in ovarian carcinomas. This becomes even more important as the P-gp related drug taxol is nowadays included in first and second line treatment of ovarian carcinomas.[1] No prognostic importance or relation with response to chemotherapy was found by us for P-gp in untreated ovarian carcinomas. In a study by Holzmayer et al the presence of even very low levels of P-gp expression correlated with lack of response to natural drugs.[24] Meta-analyses have suggested that adriamycin adds to the efficacy of cisplatin based chemotherapy.[28] The impact of P-gp expression on response to combination chemotherapy may be obscured by non-P-gp related response to platinum and cyclophosphamide. Different levels of topoisomerase II (target of adriamycin) in malignant ovarian tumors may also have a role in variable response to chemotherapy.[23,29] Another confounding factor in relating tumor response to P-gp immunostaining and also the immunostaining of GST pi, c-erbB-2, and p53 may be the fact that evaluation laparotomy in this series of patients was performed after three cycles of chemotherapy. This relatively early timing bears the danger of underestimating the frequency of responders versus non responders. However, recent studies on the course of the tumor marker CA 125 in ovarian cancer during first line chemotherapy have shown, that especially the CA 125 level after three cycles of chemotherapy is an important indicator of response (for review, see Crombach et al[30]). Therefore, it may be that some patients with partial remission after three cycles have been in complete remission after six cycles of chemotherapy, but the number of patients with stable or progressive disease after three cycles that have changed in partial remission or complete remission after six cycles most probably will be very low, and not important enough to limit the power of our statistical comparisons.

Abundant GST pi immunostaining was found by us in 79/89 (89%) of untreated ovarian carcinomas and intratumor heterogeneity was not significant. No relation of GST pi immunostaining with PFS or response to chemotherapy could be found. In a previous study using an HPLC method we found that GST pi was the predominant GST isoenzyme in ovarian carcinomas. Surprisingly, GST pi levels were lower in tumors obtained after chemotherapy and GST pi level was no prognostic indicator for response to platinum chemotherapy.[9] Because the previous study comprised a relatively small series of tumors and our HPLC detection method excluded the evaluation of tumor heterogeneity, the prognostic impact of GST pi was again evaluated in the present study. Our negative results are in agreement with another recent study on GST in ovarian carcinomas[31], but in contrast with a study by Green et al.[32] In the last study it was found that high intensity of GST pi staining in untreated ovarian carcinomas was related to resistance to cytotoxic chemotherapy and shorter overall survival. Green et al used immunostaining for semi-quantification, despite the fact that this method was performed in paraffin-embedded sections, subjected to variation in

fixation time. In our present study no induction of GST pi expression was found in individuals after chemotherapy. Our results do not identify GST pi as an important parameter of drug resistance or survival in ovarian carcinomas.

The frequency of c-erbB-2 immunostaining in the present study (18/89; 20%) is within the wide range of frequencies found in other studies (0-50%).[33-36] The use of archival paraffin-embedded material may lead to underestimation of c-erbB-2 expression.[33] However, other studies in breast and ovarian carcinomas showed comparable figures of c-erbB-2 immunostaining in paraffin-embedded and frozen sections of the same tumor.[34,35] In accordance with our study recent studies did not show any prognostic significance of c-erbB-2 immunostaining [35,36], while in earlier studies c-erbB-2 immunostaining did have negative impact on survival.[33] These conflicting results may be due to several causes such as different methods for immunostaining, subjectivity of scoring, and patient selection, which are often not well defined in the different studies. However, the presence of c-erbB-2 overexpression in an important percentage of tumors in most studies points to a role of c-erbB-2 in the growth regulation of some ovarian carcinomas. Therefore, the findings of Hancock et al, who showed that blocking of c-erbB-2 overexpression with monoclonal antibodies against c-erbB-2 resulted in enhanced cytotoxicity of cisplatin in ovarian tumor cell lines, may have clinical implications in the future.[37]

The frequency of p53 immunostaining in the present study in stage I/II (20%) and in stage III/IV (40%) ovarian carcinomas is comparable to that in other studies [38-42] The results of p53 detection with antigen retrieval using tissue unmasking fluid in paraffin-embedded sections are in complete concordance with results in frozen sections from the same tumor.[19] Immunostaining of p53 in ovarian carcinomas has been found to be strongly associated with the occurrence of missense mutations.[38] In most human malignancies such as carcinomas of the breast, lung, colon, and prostate immunostaining of p53 protein (as an indicator of mutation) is a negative prognostic factor.[14] In the present study patients with p53 positivity had significantly shorter PFS and overall survival. Log rank analysis with adjustment for trends clearly showed that p53 is not an independent prognostic factor in ovarian carcinomas. Since p53 immunostaining was associated with grade III ovarian carcinomas, the presence of ascites, and large residual tumor after first laparotomy, and not with response to chemotherapy, the negative prognostic impact of p53 immunostaining appears to be due to more aggressive tumor growth. As the aim of our study was not to evaluate the impact of each prognostic factor described in ovarian carcinomas no further multivariate analysis was performed. The first report on p53 immunostaining in a larger series of patients with ovarian carcinomas did not show prognostic significance with respect to survival.[38] Recently, two studies reported a negative prognostic role for p53 immunostaining in ovarian carcinomas.[41,42] In these studies p53 positivity was also associated with grade III tumors, but no information was available on important clinical prog-

nostic factors, such as residual tumor after first laparotomy and presence of ascites. The negative prognostic significance of p53 immunostaining presents (mutant) p53 as an interesting potential therapeutic target in ovarian carcinomas.[43] In vitro studies have shown that the P-gp gene may be activated by mutant p53.[44] In the present study p53 immunostaining was not related to P-gp staining.

In conclusion, the negative prognostic role of p53 and induction of P-gp in residual tumors after chemotherapy indicate that these cell biological parameters may have a role in the clinical course of patients with ovarian carcinoma. However, the determination of these two parameters or c-erbB-2 and GST pi at diagnosis did not aid in the prediction of response to chemotherapy. At the same time, response to chemotherapy had the strongest prognostic impact on PFS in stage III/IV ovarian carcinomas. Perhaps other parameters, related to drug resistance, such as expression of DNA repair genes[45] will be helpful in better predicting response to chemotherapy in individual patients with ovarian carcinoma.

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Chapter 8

Higher levels of interleukin-6 in cystic fluids from patients with malignant versus benign ovarian tumors correlate with decreased hemoglobin levels and increased platelet counts

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Abstract

Background *Recently high pretreatment platelet counts and low pretreatment hemoglobin have been identified as negative prognostic factors in patients with ovarian cancer. Interleukin-6 (IL-6) is a multifunctional cytokine with a diversity of functions leading to induction of C-reactive protein (CRP), increased platelet counts, and low hemoglobin. Different epithelial ovarian cancer cell lines have been found to produce varying amounts of IL-6. In this study a possible relation between IL-6 levels in cystic fluids of benign and malignant ovarian tumors and pretreatment serum CRP, platelet counts, and hemoglobin levels was evaluated.*

Methods *Bio-assay and enzyme-linked immunosorbent assay (ELISA) were used to determine the levels of IL-6 in cystic fluids and serum from patients with benign and malignant ovarian tumors.*

Results *Median IL-6 was higher in cystic fluids of malignant tumors when compared with cystic fluids of benign tumors ($p<0.01$ for both bio-assay and ELISA). Serum IL-6 levels in patients with malignant tumors were not significantly higher in comparison to IL-6 levels in patients with benign tumors, while CRP levels were higher in patients with malignant tumors ($p<0.01$). Cystic fluid IL-6 levels were related to serum CRP levels ($r=0.60$, $p<0.01$ (bio-assay); $r=0.62$, $p<0.01$ (ELISA)), platelet counts ($r=0.60$, $p<0.01$ (bio-assay); $r=0.41$, $p<0.01$ (ELISA)), and inversely related to hemoglobin levels ($r=-0.57$, $p<0.01$ (bio-assay); $r=-0.54$, $p<0.01$ (ELISA)).*

Conclusions *IL-6 levels are higher in cystic fluids of malignant ovarian tumors compared with benign tumors. The relation of cystic fluid IL-6 levels with CRP, platelet counts, and hemoglobin points to a possible causative role of tumor-derived IL-6 in the appearance of general side-effects in ovarian cancer, which have recently been recognized as prognostic factors.*

Introduction

Well known prognostic factors in epithelial ovarian cancer are performance status of the patient, differentiation grade of the tumor, amount of residual tumor after first laparotomy, and presence or absence of ascites (1). Recently Kappen et al, using a new quantitative method based on large patients numbers, identified additional predictive factors such as: pretreatment platelet count, and hemoglobin level (2). High pretreatment platelet counts and low hemoglobin levels were found to be negative prognostic factors in patients with ovarian cancer.

Interleukin-6 (IL-6) is a multifunctional cytokine that is produced by T cells, monocytes, fibroblasts, endothelial cells and keratinocytes (3). IL-6 has a diversity of functions: it acts as an hepatocyte-stimulating factor and induces the production of various acute-phase proteins such as CRP in liver cells. IL-6 induces maturation of megakaryocytes, resulting in an increase in platelets and it activates osteoclasts as well as bone resorption (3,4). In vivo studies in cancer patients treated with chemotherapy, in which recombinant human (rh) IL-6 was administered as thrombopoietic drug, confirmed the stimulating effect of IL-6 on platelet counts, but also showed an unexpected and as yet unexplained decrease in hemoglobin levels (6-8). IL-6 has also been shown to be an autocrine growth factor in human myeloma cells and to induce the in vitro proliferation as well as inhibition of several non-hematological malignant cell lines, for review, see Guillaume et al (9,10). Also different epithelial ovarian cancer cell lines have been found to produce varying amounts of IL-6 (10-12). Elevated levels of IL-6 have been detected in ascitic fluid and serum from patients with ovarian cancer (13-15).

The present study was performed to investigate a possible relation between IL-6 levels in cystic fluids of malignant and benign ovarian tumors and recently recognized prognostic factors in ovarian cancer, such as pretreatment platelet counts, and hemoglobin levels. It was found that tumor-derived IL-6 may have a role in the appearance of high platelet counts and low hemoglobin as negative prognostic factors in ovarian cancer.

Materials and methods

Tumors

Cystic fluids were collected from 42 (21 benign and 21 malignant) cystic epithelial ovarian tumors consecutively operated at the University Hospital of Groningen. Samples of cystic fluids were obtained from cystic tumors immediately after extirpation by fine-needle aspiration. Next, samples were divided into aliquots and stored at -70°C. Tumors were classified according to the WHO classification using paraffin-embedded tissue (16). Patients with malignant tumors were staged according to the International Federation of Obstetrics and Gynecology (FIGO) classification (17). Carcinomas were graded into well (grade I), moderately (grade II), and poorly (grade III) differentiated ones, as described by Sobre et al (18). Stored pretreatment serum samples for CRP determination were available from 24 patients. Pre-operative hematologic data such as hemoglobin level (g/l; normal value (females); 120-160) and platelet counts (per mm^3; normal value (females); 150,000-400,000) were obtained one day before surgery and were available in all patients (19).

IL-6 bio-assay

IL-6 biologic activity in cystic fluid and serum specimens was determined by bio-assay using the IL-6 dependent murine hybridoma cell line B9 (kindly provided by Dr. L. Aarden, CLB, Amsterdam, The Netherlands), as described by Helle et al with minor modifications (20). Briefly, B9 cells are maintained in RPMI-1640 medium (Gibco BRL, Gaithersburg, Md) supplemented with 13% fetal calf serum, 50 µM 2-mercaptoethanol, 60 µg/ml gentamycin, 2 mM glutamin, 1 mM sodium pyruvate, and 20 U/ml of rhIL-6. Duplicate heat-inactivated cystic fluid or serum samples were serially diluted in 96-well flat bottom plates (Costar, Cambridge, MA). A total of 5000 washed B9 cells was added per well, and the plates were incubated at 37°C for 72 h. Cell proliferation was determined in a colorimetric assay with tetrazolium (Sigma, St. Louis, MO). As standard rhIL-6 (3.10^8 U/mg) from Genzyme (Boston, MA) was used. Lower limit of detection was 2 U IL-6/ml serum.

IL-6 enzyme-linked immunosorbent assay (ELISA)

The IL-6 ELISA was performed as described by Helle et al with minor modifications (21). Briefly, the purified monoclonal antibody CLB.MIL6/16 (0.5 µg/ml, CLB, Amsterdam, The Netherlands) was coated for 24 h at room temperature to flat-bottomed microtiter plates (Costar 6891-S, Cambridge, MA). All subsequent incubations were in 100 µl volumes at room temperature. After washing, standards with known amounts of rhIL-6 (Genzyme, Boston, MA) and serially diluted duplicate cystic fluid or serum samples were added to the plates during 1 h. The polyclonal sheep anti human IL-6 (CLB, Amsterdam, The Netherlands) was biotynylated using

LC-biotin-N-hydroxysuccimide ester (Pierce, Rockford, IL) according to the manufacturers instructions. Without further washing an excess of biotynilated sheep anti-IL-6 was added and incubated during 2 h. Normal sheep serum (10%) was added to prevent false-positive reactivity. Plates were washed extensively with phosphate buffered saline with 0.05% Tween-20 and incubated with streptavidin-horseradish peroxidase (HRPO)(Dakopatts A/S, Copenhagen, Denmark) for 30 min. Signal amplification was performed using catalyzed reporter deposition (CARD). After extensive washing plates were incubated for 15 min with biotin-tyramin, prepared as described by Bobrow et al(22). Plates were washed and again incubated with streptavidin-HRPO. After washing the plates were developed using ortho-phenyldiamine during 15 min. The reaction was stopped by the addition of an equal volume of 2 M H_2SO_4 to the wells. Plates were read at 492 nm in a Titertek Multiskan reader. The detection range of the assay was 3-400 pg/ml with a lower detection limit of 6 pg/ml IL-6 in serum.

CRP ELISA

The CRP ELISA was performed as previously described (23). In brief, rabbit anti-human-CRP (DAKO, Glostrup, Denmark) was used as coating and HRPO-conjugated rabbit anti-human-CRP (DAKO, Glostrup, Denmark) as detecting antibody in a standard sandwich ELISA procedure. As standard the CRP reference serum from Behring (Warburg, Germany) was used; lower limit of detection: 1 ng/ml.

Statistical analysis

The Wilcoxon test for two groups of unpaired observations, chi square analysis for frequency tables, and the Spearman rank analysis were used for statistical analysis. P values < 0.05 were considered significant.

Results

Tumors

Cystic fluids were obtained from 21 benign epithelial ovarian tumors (12 serous and 9 mucinous cystadenomas), and from 21 malignant epithelial ovarian tumors (7 serous, 4 undifferentiated, 3 endometroid, 4 mucinous, and 3 clear cell adenocarcinomas). Two patients had a malignant ovarian tumor FIGO stage I, 2 patients FIGO stage II, 14 patients FIGO stage III, and 3 patients FIGO stage IV disease. Eight patients had grade I, 7 patients grade II, and 6 patients grade III ovarian cancer.

Cystic fluid IL-6 levels

Fig. 1 shows that a strong correlation was found between the results of the IL-6 levels

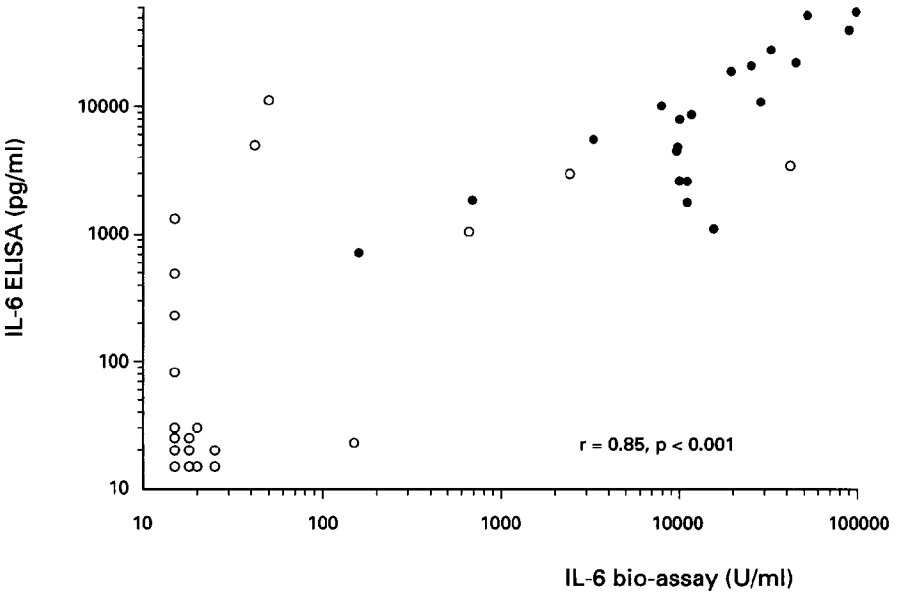

Figure 1 Levels of IL-6 (bio-assay and ELISA) in cystic fluids of ovarian tumors (○ = benign tumors, ● = malignant tumors) and the relation between both assays.

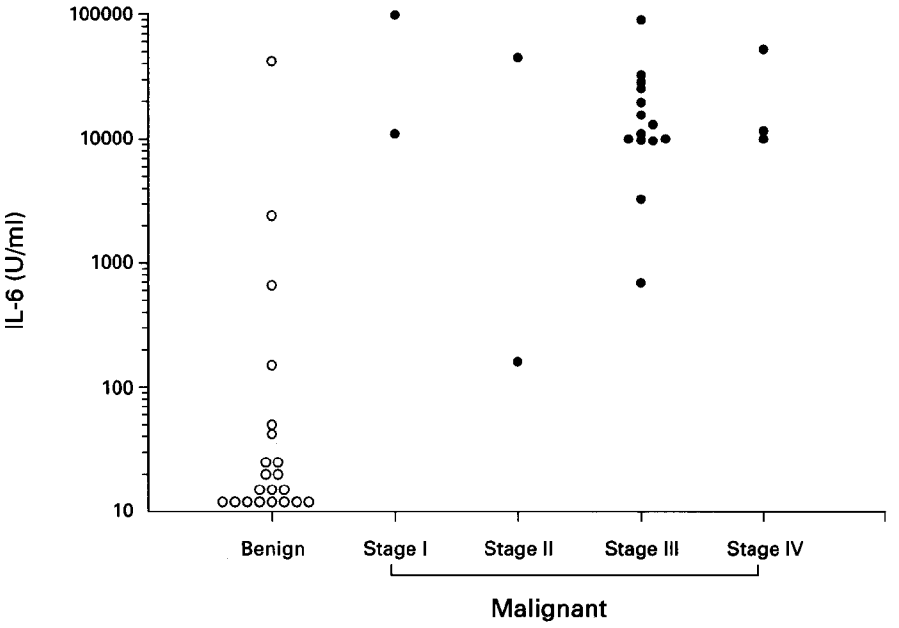

Figure 2 Levels of IL-6 (bio-assay) in cystic fluids of benign (○) and malignant (●) ovarian tumors, subdivided according to FIGO stage.

as determined by bio-assay and ELISA (r=0.85, p<0.01). In both assays median IL-6 was significantly higher in the cystic fluids of malignant tumors (IL-6 bio-assay; 11,600 U/ml (range 690-98,500), IL-6 ELISA; 8,681 pg/ml (range 160-55,820)) in comparison to median IL-6 in the cystic fluids of benign tumors (IL-6 bio-assay; 10 U/ml (range <2-42,000), IL-6 ELISA; 10 pg/ml (range <6-4953) (see fig. 1 and fig. 2). When the IL-6 levels in the malignant tumors were analyzed separately, no relation was found with tumor stage or grade (see fig. 2, IL-6 versus tumor stage).

Serum IL-6 and CRP levels

IL-6 activity was detectable by bio-assay in 4/11 sera from patients with benign tumors and 8/13 sera from patients with malignant tumors. Median serum IL-6 in patients with benign tumors (5 U/ml, range <2-20) was comparable to median serum IL-6 in patients with malignant tumors (12 U/ml, range <2-19). Using the IL-6 ELISA no IL-6 was detectable. No relation was found between serum IL-6 levels (bio-assay) and IL-6 levels (bio-assay and ELISA) in cystic fluids. CRP was detectable in 3/11 sera from patients with benign tumors and 12/13 sera from patients with malignant tumors (p<0.05). Median serum CRP (18 mg/l, range <1-117) was higher (p<0.01) in patients with malignant tumors in comparison to median serum CRP in patients with benign tumors (<1 mg/l, range <1-7).

Table 1 Relation between cystic fluid IL-6 (bio-assay / ELISA) levels and serum CRP, hemoglobin, and platelet counts.

	n	IL-6 (bio-assay)	IL-6 (ELISA)
All tumors			
CRP	24	r= 0.60[c]	r= 0.62[c]
hemoglobin	42	r=-0.57[c]	r=-0.54[c]
platelet counts	42	r= 0.60[c]	r= 0.41[a]
Malignant tumors			
CRP	13	r= 0.38[d]	r= 0.27[d]
hemoglobin	21	r=-0.57[b]	r=-0.36[d]
platelet counts	21	r= 0.46[a]	r= 0.18[d]

[a] p< 0.05; [b] p< 0.02; [c] p< 0.01; [d] not significant.

Relations between cystic fluid IL-6 (bio-assay and ELISA) and serum CRP, platelet counts and hemoglobin

Table 1 summarizes the relations between cystic fluid IL-6 (bio-assay and ELISA) levels and serum CRP, platelet counts and hemoglobin. Fig. 3, 4, and 5 show that, when analyzing all tumors, cystic fluid IL-6 levels (bio-assay) were related to serum

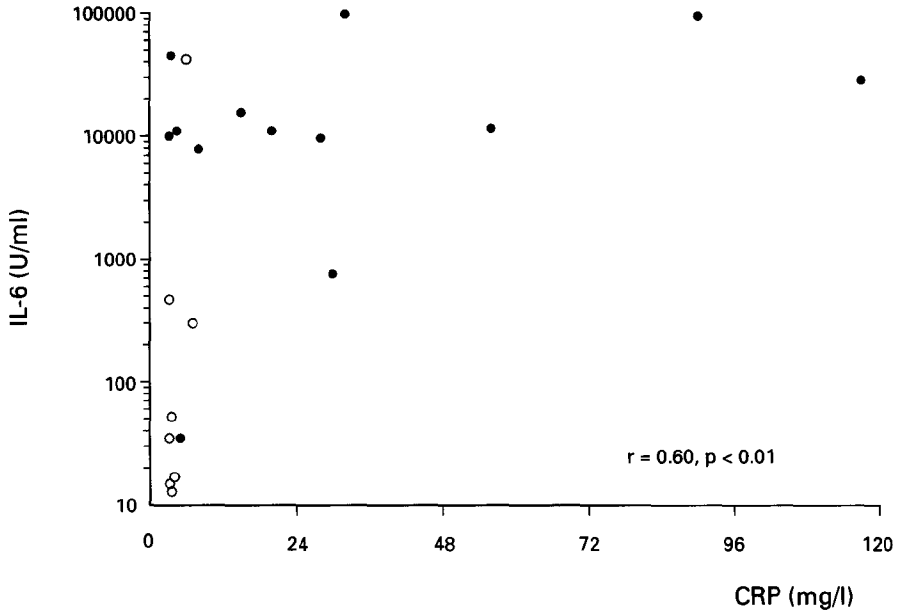

Figure 3 Cystic fluid (○ = benign tumors, ● = malignant tumors) levels of IL-6 (bio-assay) in relation to serum CRP levels.

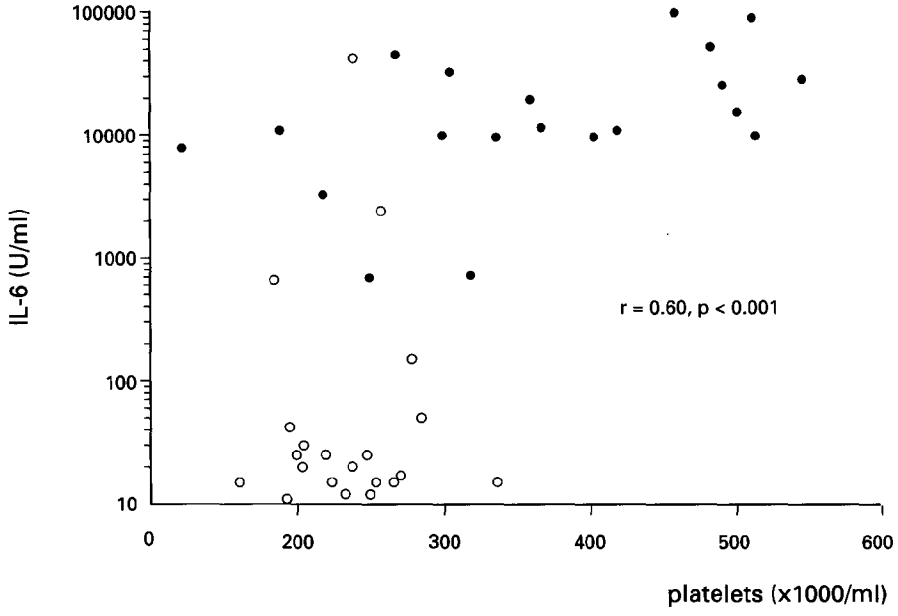

Figure 4 Cystic fluid (○ = benign tumors, ●= malignant tumors) levels of IL-6 (bio-assay) in relation to platelet counts.

CRP levels, platelet counts, and hemoglobin levels. When the malignant tumors were analyzed separately no relation was found between cystic fluid IL-6 levels and serum CRP. IL-6 levels as determined by bio-assay still were related to platelet counts and hemoglobin levels (see table 1). However, no relation was found between IL-6 levels as determined by ELISA and platelet counts or hemoglobin (see table 1).

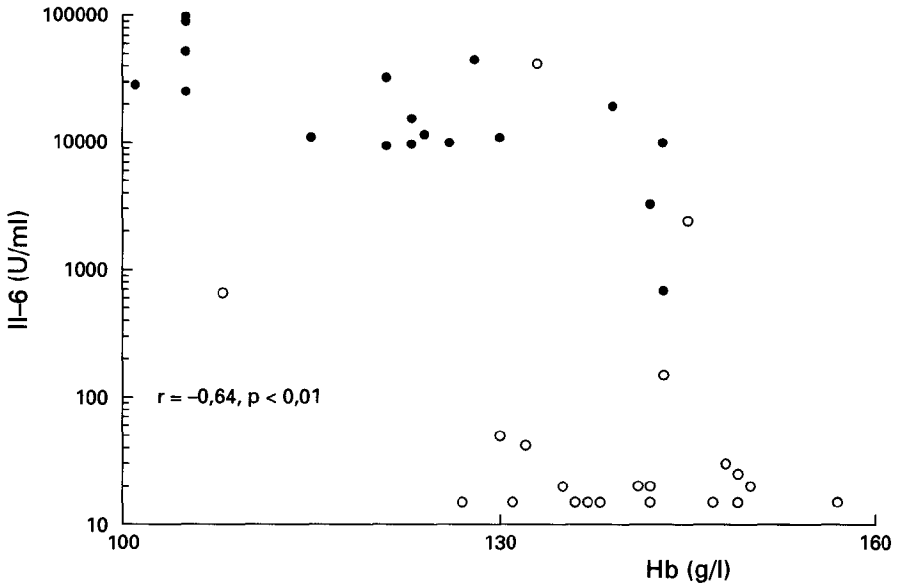

Figure 5 Cystic fluid (○ = benign tumors, ● = malignant tumors) levels of IL-6 (bio-assay) in relation to hemoglobin.

Discussion

Our study shows higher IL-6 levels in cystic fluids from malignant ovarian tumors in comparison to benign ovarian tumors. Several ovarian cancer cell lines and primary ovarian tumor cultures have been found to produce and secrete IL-6 (10-12). Previous studies reported increased IL-6 levels in ascites of patients with malignant ovarian tumors in comparison to IL-6 levels in peritoneal fluids from non-cancer patients (14,15). However, IL-6 in ascites may also have its origin in mesothelial cells lining the peritoneal cavity and / or peritoneal leukocytes, which have been shown to be able to produce IL-6 (16). IL-6 levels (determined by ELISA) in cystic fluids of malignant tumors (median: 8681 pg/ml, range 160-55,820) as found in the present study appear to be somewhat higher than IL-6 levels in ascites from patients with malignant tumors

(median: 4,291 pg/ml, range 408-8,908), which IL-6 levels were recently reported by Moradi et al (15). Higher IL-6 levels in cystic fluids in comparison to ascites support the hypothesis that IL-6 in ascites is (in part) produced by ovarian tumors, although our methods do not discriminate between IL-6 produced by ovarian tumor cells or tumor-associated macrophages (24,25).

In 8/13 patients with malignant ovarian tumors we found serum IL-6 activity by bio-assay, while no serum IL-6 was detectable by ELISA. With concern to the bio-assay these figures are comparable to the results of Berek et al who found detectable IL-6 activity in serum of 16/21 patients with advanced stage ovarian cancer (13). Moradi et al found detectable serum IL-6 levels by ELISA in 15/17 patients with untreated ovarian cancer (15). In their study a surprisingly high mean level of IL-6 (109.7 pg/ml) was found in sera of control patients. No relation was found by us between IL-6 levels in cystic fluids and sera. Except for the small number of evaluable patients (n=24) another explanation may be the small range in the relatively low serum IL-6 levels. Moreover, serum levels of IL-6 are not only determined by the production of IL-6, but also by adsorption of IL-6 by different cells with an IL-6 receptor.

In our study IL-6 levels in cystic fluids of the malignant tumors were not related to grade or stage of the tumors. Recently, Lidor et al showed that normal and malignant cultured epithelial ovarian cells both produce comparable levels of IL-6 (26). The higher IL-6 levels in cystic fluids from patients with malignant tumors in comparison to benign tumors are therefore most likely due to the fact that the number of tumor cells producing IL-6 is much higher in the cystic wall of the malignant tumors in comparison to the number of tumor cells in the normally thin layer of epithelial cells in the cystic wall of benign tumors. As the exact role of IL-6 in growth regulation of ovarian epithelium remains to be determined there is no obvious reason for a relation of cystic fluid IL-6 levels with tumor grade or stage as parameters of more aggressive tumor growth.

IL-6 has been found to induce the production of acute phase proteins such as CRP by hepatocytes (4) and therefore the level of CRP may be regarded as the endproduct of IL-6 stimulation. Recently, Berek et al reported a relation between IL-6 levels in ascites and serum CRP in patients with ovarian cancer (14). These findings are confirmed in the present study where IL-6 levels in cystic fluids of epithelial ovarian tumors correlated with serum CRP levels, most likely due to leakage of IL-6 to the circulation. RhIL-6 has also been found both in vitro and in vivo to increase platelet counts by the stimulation of megakaryocytes(4) which is reflected in the present study by the relation between high IL-6 levels in cystic fluids and high platelet counts. The inverse relation between high IL-6 levels in cystic fluids and low hemoglobin levels is in accordance with studies in which the administering of rhIL-6 as trombopoietic growth factor lead to an unexplained decrease in hemoglobin levels (6-8). Serum CRP, platelet counts, and hemoglobin levels appear from our study as in vivo bio-

assays of IL-6 production by ovarian tumors. There are however some major drawbacks: we evaluated the concentration of IL-6 in the cystic fluids and did not take in account the absolute volume of cystic fluid; only the relation of IL-6 levels as determined by bio-assay with hemoglobin and platelet counts remained significant when malignant tumors were analyzed separately. This lack of relation may be due to the small number of patients with malignant tumors (n=21). Another explanation for the relation of IL-6 levels in all cystic fluids with the different in vivo bio-assays may be, that higher IL-6 levels, serum CRP and platelet counts and low hemoglobin levels in the malignant tumors are coincidential, and merely a reflection of other differences between benign and malignant tumors. For example, low hemoglobin levels might very well be the result of poor nutrition in these patients with advanced ovarian cancer. Therefore, a larger study must be performed to confirm the causative role of IL-6 in the appearance of general side-effects in ovarian cancer patients. In vivo and in vitro data in other diseases than ovarian cancer have established the role of IL-6 in the appearance of general side-effects such as thrombocytosis, anemia, and fever (27-29). If in future studies a similar role of IL-6 is confirmed in patients with ovarian cancer the administration of neutralizing antibodies to IL-6 may result in normalization of platelet counts and hemoglobin levels and improvement of the performance status of the patient, as was found for neutralizing anti IL-1 and tumor necrosis factor α antibodies in mice (30). As performance status is an important prognostic factor in ovarian cancer the reversal by neutralizing antibodies of general side-effects caused by tumor-derived IL-6 may have important clinical implications in the management of patients with ovarian cancer.

Apart from a role in the generation of general side-effects in ovarian cancer patients it remains as yet unclear if IL-6 has a role as (autocrine or paracrine) growth promoting factor in ovarian tumor cells. Tamm et al have found that IL-6 decreased cell-cell association and increased the motility of ductal breast carcinomas cells (31). Recently, we observed a relation between p53 overexpression and negative prognostic factors such as large residual tumor before chemotherapy and presence of ascites in a study in advanced ovarian cancer (32). Similar observations have been reported by other groups (33,34). Wild type p53 has been reported to repress the transcription of IL-6, which ability is lost by mutant p53 (35,36). Currently, we are performing studies in human ovarian carcinoma cell lines and tissues to analyze the relation between p53 expression and IL-6 expression and/or production by malignant ovarian tumor cells.

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Chapter 9

Prognostic value of the MDR associated protein LRP in advanced ovarian carcinomas

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Abstract

The expression and prognostic value of lung resistance rotein (LRP) (a recently identified protein associated with the non-P-glycoprotein multidrug resistance phenotype) and P-glycoprotein were evaluated in patients with advanced-stage ovarian carcinoma with respect to response to chemotherapy and (progression free) survival. Immunostaining of LRP and P-glycoprotein was performed on frozen sections of 66 stage III/IV primary tumors using monoclonal antibody LRP-56 and two P-glycoprotein-specific monoclonal antibodies, respectively. Fifty-seven patients received chemotherapy (platinum based in 88%) after surgery. The results of immunostaining were related to clinicopathologic prognostic factors in ovarian carcinoma, response to chemotherapy and (progression free) survival. LRP and P-glycoprotein immunoreactivity were present in respectively, 74% and 13% of cases. LRP expression tended to be negatively associated with high tumor grade. LRP, but not P-glycoprotein immunostaining was associated with complete response to induction chemotherapy (50% complete responses in tumors with negative LRP staining versus 8% complete responses in tumors with positive LRP staining). Patients with positive immunostaining for LRP in the tumor had a shorter progression free and overall survival in comparison to patients with negative immunostaining. P-glycoprotein immunostaining at diagnosis had no prognostic value. These results indicate that LRP, but not P-glycoprotein, defines a drug resistant phenotype expressed intrinsically in this group of patients with ovarian carcinoma. Moreover, LRP has a strong independent negative prognostic significance with regard to progression free and overall survival in these patients.

Introduction

The majority of patients with ovarian carcinoma present with advanced-stage disease that has metastasized throughout the peritoneal space. Therefore, radical surgical excision of all tumor is often not possible and patients have to be cured by chemotherapy. Standard chemotherapy consists of platinum containing drugs in combination with alkylatings agents and/or doxorubicin. Response rates to these regimens vary between 60-70%, but the complete response rate as judged by pathological examination at second look laparotomy after six cycles of standard chemotherapy is only 20-30%. Response rates of residual or recurrent tumors to chemotherapy after first line treatment are disappointing (0-40%), resulting in the poor survival figures of patients with advanced-stage carcinoma (5-years survival: 10-20%)(1). From these clinical data it appears that intrinsic or acquired resistance to chemotherapy is the major obstacle in the management of patients with advanced-stage disease.

Well-known prognostic factors in ovarian carcinoma are advanced-stage of disease, presence of bulky residual disease after initial surgery, older age, bad performance status, high tumor grade, and presence of clear cell or mucinous adenocarcinoma (2). More recently, tumor DNA ploidy, and pre- and postoperative serum CA 125 levels have been proposed as independent prognostic factors in ovarian carcinoma (3,4). Combinations of these prognostic factors can adequately predict the long-term survival of patients before initiation of chemotherapy. However, prediction of response to chemotherapy in individual patients is not possible (5). New prognostic factors specifically related to response to chemotherapy could have additive value in this respect.

Studies in cultured (ovarian) tumor cells have revealed a variety of mechanisms at different cellular levels that may be involved in resistance to chemotherapy. P-glycoprotein (P-gp) is a cell membrane glycoprotein acting as efflux pump for certain classes of unrelated drugs, such as doxorubicin and epipodophyllotoxins (natural products). The overexpression of P-gp in tumor cell lines selected for resistance to a single natural product is accompanied by cross-resistance to other natural products, resulting in the so-called multidrug resistance (MDR) phenotype (6). Data on the frequency of P-gp expression in untreated ovarian carcinoma and its prognostic significance are controversial (7-13).

Recently we have described a M_r 110,000 lung resistance protein (LRP), that was found to be overexpressed in several non-P-gp tumor cell lines of different histogenetic origin with ATP-dependent drug accumulation defects (MDR phenotype). LRP is specifically recognized by monoclonal antibody (Mab) LRP-56, obtained after immunization of Balb/c mice with a non-P-gp MDR human lung cancer cell line (14). In a series of 61 drug unselected human cancer cell lines used at the National Cancer Institute for screening of new drugs, LRP correlated better than P-gp with in vitro in-

trinsic drug resistance. These in vitro data suggest that the mechanism of LRP-associated MDR is involved in intrinsic and early steps of acquisition of drug resistance (Izguierdo et al, submitted).

The aim of the present study was to relate immunostaining of P-gp and LRP to conventional clinicopathologic prognostic parameters in ovarian carcinomas as well as to response to chemotherapy and (progression free) survival.

Materials and methods

Patients

Freshly frozen samples (stored in liquid nitrogen until use) from 66 newly diagnosed patients undergoing surgery for advanced-stage ovarian carcinoma were obtained from diagnostic histopathology laboratories at the Free University Hospital, Amsterdam (period 1984-1993), and the University Hospital Groningen (period 1991-1993). No selection criteria were applied. Final evaluation date of the follow-up of the patients was 1 May 1994. All patients were initially treated with optimal tumor reductive surgery, and were staged according to the International Federation of Obstetrics and Gynecology (FIGO) classification (15). Data on performance status were available in 28 patients, and therefore this possible prognostic factor was not further evaluated. Residual tumor after first laparotomy was categorized as follows: residual tumor smaller than 2 cm and residual tumor larger than 2 cm in diameter. Newly diagnosed patients with epithelial ovarian carcinoma FIGO stage III and IV were eligible for this study. Table 1 summarizes the chemotherapy regimens in this group of patients. Fifty-seven patients received chemotherapy, which was platinum-based in 50 patients. Nine patients did not receive chemotherapy after surgery for various reasons such as older age and bad performance status, and were excluded from response and survival analysis. Evaluation relaparotomy was performed after six cycles of chemotherapy in 30 patients. Tumor response to chemotherapy was classified according to WHO criteria only in patients with evaluable disease after first laparotomy (16). Patients with incomplete response to first line chemotherapy and patients with recurrent tumors were treated with a variety of second line chemotherapeutic regimens. After chemotherapy and surgery, all patients are followed with gradually increasing intervals. Progression free and overall survival were calculated in months from the day of first laparotomy.

Histology

Primary tumors were classified according to the WHO classification using paraffin embedded tissue (17). Carcinomas were graded into well (grade I), moderately (grade II), and poorly (grade III) differentiated, as described by Sobre et al(18).

Antibodies

For LRP immunostaining the previously described LRP-56 Mab was used. Mab LRP-56 was obtained after Balb/c mice immunization with the non-P-gp MDR human lung cancer cell line 2R120 in our own laboraratory (14). For P-gp immunostaining the JSB-1 and MRK16 Mabs were used as previously described (19). JSB-1 is a murine monoclonal subclass IgG1 from our own laboratory (20). MRK-16 (subtype IgG2) is described and provided by Hamada and Tsuruo (21).

Immunohistochemistry

Staining was performed on cryostat sections with the indirect immunoperoxidase method for Mab LRP-56 and with an avidin-biotin complex procedure for Mabs JSB-1 and MRK16, as previously described (22,23). Staining with an irrelevant mouse IgG was routinely performed as a negative control procedure. Cytospin preparations of LRP and P-gp positive cell lines were used for validating and controlling the immunohistochemical assays.

The slides were examined and scored independently by two of us (M.A.I. and P.v.d.V) without knowledge of the clinical data of the patients. In selected cases with equivocal results the staining was repeated to ensure reproducibility. By prior agreement, for LRP expression staining was defined as positive if more than 10% of the tumor cells showed positive staining. For P-gp a tumor was considered to be positive if more than 10% of tumor cells stained with both, JSB-1 and MRK16 Mabs.

Statistical methods

A computer file was compiled of the data for each patient and the data were analyzed using the BMDP statistical software package (BMDP Statistical Software, Inc, Los Angeles, CA). Clinicopathological parameters determined at diagnosis were assessed for their relationship with LRP and P-gp expression using chi-square analysis or Fisher's exact 2-tailed test, when appropiate. The duration of (progression free) survival was estimated in months by the method of Kaplan-Meier. Differences in (progression free) survival from the first laparotomy in the diverse subgroups were analyzed using log-rank statistics. Multivariate analysis was performed with a Cox proportional-hazards regression model, including all the factors that were significant in the univariate analysis. Hypotheses were evaluated at a significance level of 0.05. Unless otherwise specified, all P values are two-sided.

Results

Patients.

The first two columns of table 1 summarize the clinicopathologic data of our series.

Table 1 Characteristics of 66 advanced ovarian carcinoma patients at diagnosis in relation to LRP expression.

		No. of LRP - tumors	No. of LRP + tumors	P-value[1]
Total no. of patients		17(26)[2]	49(74)	
Age	≤ 66	6(35)	27(55)	0.26
	> 66	11(65)	22(45)	
FIGO Stage	III	11(65)	36(73)	0.54
	IV	6(35)	13(27)	
Residual tumor	≤ 2cm	8(47)	17(35)	0.39
	> 2cm	9(53)	32(65)	
Histology				
	SA	11(64)	34(70)	0.13
	MA	0	8(16)	
	AC	4(24)	5(10)	
	others	2(12)	2(4)	
Tumor grade	I/II	3(18)	21(43)	0.08
	III	14(82)	28(57)	
Ascites[3]	yes	7(41)	33(61)	0.55
	no	10(59)	14(29)	

SA: serous adenocarcinoma; MA: mucinous adenocarcinoma; AC: unspecified adenocarcinoma; [1]: chi-square/Fisher's exact test; [2]: (percentage); [3]: 64 patients were evaluable for ascites, in two patients information was not available.

Median age of the patients at diagnosis was 66 years (range: 29-84). Median follow-up of the 57 patients included in the survival was 18 months (range: 1-111 months). Thirty-eight patients died during follow-up after first line chemotherapy. At the final evaluation date of the study 19 patients were alive (median follow-up: 31 months, range:7-111 months). The median progression free survival was 11 months (range: 1-57). The median overall survival was 18 months (range:1-111). In 30 patients a second look laparotomy was performed; 7 patients had achieved a complete pathological remission, while 23 patients had residual tumor after six courses of chemotherapy. Nine of the 66 patients did not receive chemotherapy and were excluded from the survival analysis. Eight of the 57 patients who received chemotherapy had no clinical parameters to evaluate response after first laparotomy, while no second look laparotomy was performed. Therefore, these patients were regarded as non-evaluable for response.

Table 2 First line chemotherapy regimens in 66 patients with stage III/IV ovarian carcinoma in relation to LRP expression.

	LRP negative (n=17)	LRP positive (n=49)
No chemotherapy	4(24)	5(11)
Chemotherapy	13(76)	44(89)
Platinum based	12(92)	38(86)[1]
CDDP+Cy	5	15
Ca+Cy	7	15
Ca+CDDP	0	2
CDDP+Dox/Epi±Cy	0	5
Ca	0	1
Non-Platinum based	1(8)	6(14)[1]
Me	1	5
Le	0	1

CDDP: cisplatin; Cy: cyclophosphamide; Ca: carboplatin; Dox: doxorubicin; Epi: epirubicin; Me: melphalan; Le: leukeran. [1]: Fisher's exact test, p=0.90.

Table 3 Tumor response to first line chemotherapy in 57 stage III/IV ovarian carcinoma patients in relation to LRP and P-gp expression.

Resp	LRP- (n=13)	LRP+ (n=44)	P-value[1]	P-gp- (n=48)	P-gp+ (n=9)	P-value[1]
CR	5(50)[2]	3(8)		8(20)	0	
PR	3(30)	14(36)	0.004[3]	13(33)	4(44)	NS
NR	2(20)	22(56)		19(47)	5(56)	
NE	3	5		8	0	

Resp: response to chemotherapy; CR: complete response; PR: partial response; NR: no response; NE: not evaluable for response; [1]: chi=square test; [2]:(percentage); [3]: CR+PR versus NR.

Immunohistochemistry.

Table 1 summarizes LRP staining results in relation to clinipathologic parameters. Of the 66 patients studied 49 (74%) and 9 (13%) were considered to be LRP and P-gp positive, respectively. The LRP staining pattern was coarsely cytoplasmatic in the majority of tumors. Patients in the LRP negative and positive expression groups had similar clinicopathologic features except for tumor grade. There was a tendency

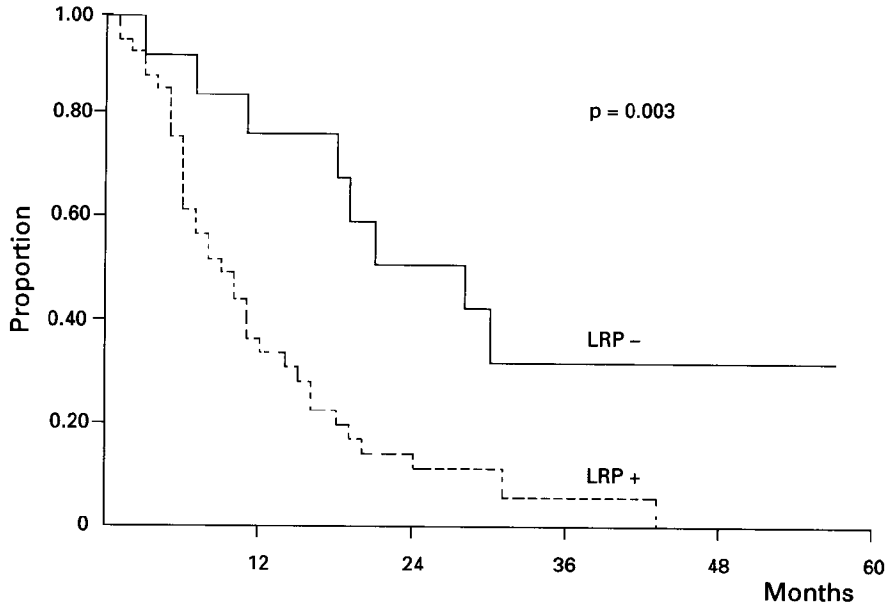

Figure 1 PFS for stage III/IV ovarian carcinomas by LRP staining characteristics.

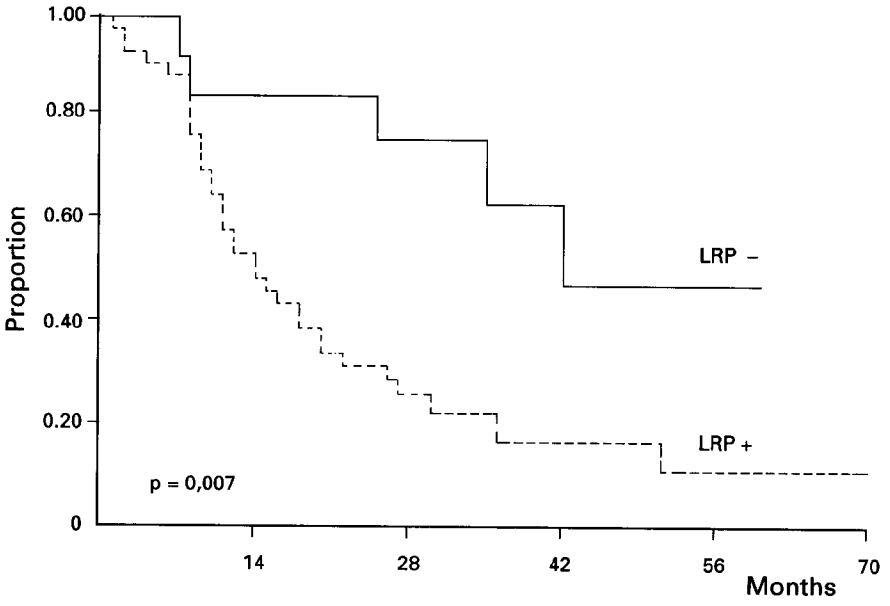

Figure 2 Overall survival for stage III/IV ovarian carcinomas by LRP staining characteristics.

Table 4 Univariate analysis for 57 stage III/IV ovarian carcinoma patients treated with chemotherapy. P-values from the log-rank analysis are shown[1].

		Progression free survival	Overall survival
Age	≤ 66 (n=32) > 66 (n=25)	0.87	0.54
FIGO Stage	III (n=43) IV (n=14)	0.05	0.03
Residual tumor	≤ 2cm (n=24) > 2cm (n=33)	0.07	0.04
Histology	SA (n=40) other types(n=17)	0.10	0.24
Tumor grade	I/II (n=21) III (n=36)	0.18	0.02
Ascites[2]	yes (n=37) no (n=19)	0.04	0.06
P-gp	negative (n=48) positive (n=9)	0.77	0.70
LRP	negative (n=13) positive (n=44)	0.003	0.007

[1]: P-value < 0.05 means significant difference in progression free or overall survival between the two groups; [2]: no information available in one patient; SA: serous adenocarcinoma.

towards more undifferentiated tumors in the LRP negative group. Identical results were obtained for the 57 patients treated with chemotherapy when evaluated for response and survival (data not shown). Table 2 summarizes the chemotherapy regimens according to the LRP expression groups. No differences regarding chemotherapy were found for the two groups except that six patients in the LRP positive group received doxorubicin or epirubicin as part of their chemotherapy regimen.

Response to chemotherapy

The correlation between LRP and P-gp expression and response to chemotherapy was evaluated in 49 patients who received chemotherapy and were evaluable for response (for summary, see table 3). Positive LRP immunostaining was significantly correlated with no response to chemotherapy. In particular, patients with positive LRP immunostaining had less chance to achieve (pathological and/or clinical) complete remission and higher chance of non-responding to chemotherapy ($p<0.004$). When only patients

treated with platinum-based regimens were analyzed the correlation remained significant (data not shown). There was no correlation between P-gp expression and response to chemotherapy. However, none of the patients with P-gp positive tumors achieved complete remission compared with 20% of the P-gp negative group.

Survival analysis

Table 4 summarizes the results of the univariate analysis for progression free and overall survival. All 57 patients treated with chemotherapy were included in the analysis. FIGO stage, residual tumor after first laparotomy, tumor grade, and presence or absence of ascites had significant or marginal significant impact in progression free and overall survival. Patients with LRP positive tumors has shorter progression free and overall survival (p=0.003 and 0.007)(see Fig. 1 and 2). This correlation remained significant when only patients treated with platinum-based regimen were included (data not shown). No differences were found between P-gp expression groups. In this group of 57 patients only positive LRP staining retained its prognostic significance with regard to (progression free) survival in multivariate analysis.

Discussion

In this immunohistochemical study on frozen sections we found LRP expression (defined as positive immunostaining in > 10% of tumor cells) in 49/66 (74%) untreated ovarian carcinomas. Our study is the first report on immunostaining of LRP in ovarian carcinomas, and shows that patients with LRP positive tumors have a poorer response to (platinum containing) chemotherapy, and shorter progression free and overall survival. Positive LRP immunostaining was the most significant negative prognostic factor in univariate analysis and also was the only prognostic factor that retained its significance in multivariate analysis, thus reflecting its independent value. Loss of independent prognostic significance in multivariate analysis of other well known prognostic factors in ovarian carcinoma e.g. FIGO stage, residual tumor after first laparotomy, and presence of ascites is probably due to the fact that LRP staining is such a strong prognostic factor in our study, and also due to the relative small number of patients.

The majority of patients in this study was treated with platinum containing drugs for which no association with the MDR-1 and/or LRP phenotype is described. Still a strong relation of LRP expression and response to (platinum containing) chemotherapy was found in this series of patients. Recently, we were able to clone and sequence the gene encoding for LRP, thus providing new insights with regard to its possible functional role. The LRP gene shows a strong homology (55% identity) with the major vault protein α, cloned from the lower Dictyostelium discoideum (24,

Scheffer et al, unpublished data). Vaults are multi-subunit cytoplasmatic particles that are well preserved through evolution and present in a broad range of cell types, suggesting that they play a prominent role in fundamental cell processes. About five percent of the vaults/LRP are localized in pore complexes of the nuclear membrane, while the remaining vaults/LRP are in the cytoplasm. The proposed function of these nuclear pore complexes is to control nucleo-cytoplasmatic transport of a wide range of substrates. The function of the cytoplasmatic vaults/LRP is presently unknown, although they appear to be associated with vesicles (24,25). The observed granular cytoplasmatic staining pattern (in LRP positive tumor cell lines and human ovarian carcinomas) also suggests an association of LRP with cytoplasmatic vesicles. It is tempting to speculate that vaults/LRP may play a role in drug resistance by regulating the transport of cytotoxic drugs from the cytoplasm to the nucleus and inside vesicles. In support of this view, both decreased nuclear/cytoplasmatic ratio of drugs, and entrapping of drugs into exocytic vesicles have been described in some LRP expressing non-P-gp tumor cell lines with the MDR phenotype (26,27). However, most LRP expressing tumor cell lines with the MDR phenotype show no or only little cross-resistance to platinum containing drugs. The strong relation of positive LRP immunostaining and response to platinum containing chemotherapy in the present study raises the question of the role of LRP in resistance to platinum containing compounds. It may be that in intrinsically resistant ovarian carcinomas LRP is co-expressed with sofar undefinied genes that confer resistance to different types of drugs, including platinum compounds. Alternatively, the almost significant association of positive LRP immunostaining with high tumor grade indicates that LRP may be associated with more aggressive biological behavior of malignant tumors. Recently, strong negative prognostic value of positive LRP expression has also been shown in acute myeloid leukemia (28), while studies in other types of malignancies are in progress.

In the present study positive P-gp immunostaining was found in 9/66 (13%) of untreated ovarian carcinomas. Data on the frequency of P-glycoprotein expression in human ovarian carcinoma and its possible prognostic significance are conflicting (7-12). In a recent retrospective immunohistochemical study on paraffin-embedded sections positive P-gp immunostaining was found in 15% of untreated ovarian carcinomas (12). Other studies, using different methodologies for P-gp detection, reported lower (6 and 7%), but also higher (80 and 73%) expression rates of P-gp in ovarian carcinomas using immunohistochemistry Northern blot, and polymerase chain reaction (PCR), respectively (7-11). Higher incidences of P-gp expression were found in studies using PCR, which is a very sensitive detection technique (9,10). The wide range in reported frequencies of P-gp expression in ovarian carcinomas is obviously due to the different sensitivity of the detection techniques used. In some human malignancies elevated expression of P-gp has been linked to unresponsiveness to (MDR

related) chemotherapy and dismal prognosis (29). In the present study no relation was found of P-gp immunostaining with response to chemotherapy and (progression free survival). Recently, analogous results were reported in a study on P-gp expression in relation to response to cisplatin and doxorubicin containing chemotherapy in advanced-stage ovarian carcinomas (12). Holzmayer et al found high expression (determined by PCR of P-gp to be related to failure to respond to chemotherapy, which was even more apparent in patients treated with MDR related drugs (10). Using reverse transcription-PCR Arao et al did not find a relation between P-gp expression levels and response to chemotherapy (11). However, interpretation of both studies is hampered by the fact that the clinical information is incomplete and patient selection undefined, which weakens the strength of the observations. Overall, our own data and those from other studies indicate that P-gp expression is not a prognostic factor in patients with advanced-stage ovarian carcinoma treated with platinum containing chemotherapy. Previous treatment with natural drugs such as doxorubicin induces P-gp expression in residual tumors after chemotherapy. Therefore, overexpression of P-gp in these tumors may have clinical importance, especially when taxol (a natural product) will be more extensively used in first and second line treatment (8,12,13).

In conclusion, our results show that determination of LRP and not P-gp expression may be a useful test to predict response to chemotherapy and outcome in advanced-stage ovarian carcinomas. LRP expression is the first cell biological parameter reported in advanced ovarian carcinomas to be related specifically to response to platinum containing chemotherapy. However, the mechanism responsible for this relation remains to be elucidated. Based on the results of this retrospective study a prospective study is definitively warranted.

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Chapter 10

Summary, conclusions and perspectives

Intrinsic or acquired resistance to cytotoxic drugs is the major obstacle in the management of patients with advanced-stage ovarian carcinoma, and is therefore responsible for the poor outlook of these patients (5-year survival: 10-20%). Studies in cultured (ovarian) tumor cells have provided insight in the diverse mechanisms that may be involved in drug resistance (for summary, see Introduction).

Despite the fact that knowledge on the diverse cell biological factors involved in drug resistance has developed rapidly, the translation of this knowledge into the clinic is only just beginning. In this thesis diverse cell biological characteristics of ovarian carcinomas are studied. These cell biological factors may have an important role in drug resistance and (to less extent) tumor aggressiveness which obviously are two different entities.

In **chapter 1** a review is given of the reported genetic changes in ovarian carcinomas in possible relation to drug resistance. As spontaneous mutations in tumor cells occur approximately one in 10^4 - 10^6 cell divisions, the chances for a drug resistant mutation are directly related to the size of a tumor. Therefore, at initial surgery for ovarian carcinoma every effort should be taken to remove as much tumor as possible. In ovarian carcinoma high frequency of chromosomal loss has been observed especially at chromosome arms 6p, 17p, and 17q. No data exist on a possible relation of numerical chromosomal changes and sensitivity to cytotoxic drugs. Overexpression of the c-erbB-2 oncogene and the epidermal growth factor receptor (EGF-R) have been reported in ovarian carcinoma in approximately 20-30% and 50-60%, respectively. Most studies show that c-erbB-2 and EGF-R overexpression may have a role in growth regulation in an important percentage of ovarian carcinomas. The administration of blocking monoclonal antibodies against these growth factor receptors in combination with cisplatin may lead to additive cytotoxicity. Upregulation of the c-fos, c-jun, and c-myc oncogenes, which are nuclear transcription factors, occurs after exposure of cultured tumor cells to cytotoxic drugs. Enhanced expression of these oncogenes results in increased levels of DNA damage repair enzymes, responsible for resistance to cytotoxic drugs that have DNA as their target. The role of the c-fos, c-jun, and c-myc oncogenes in the clinical course of ovarian carcinoma, especially with regard to response to chemotherapy, remains to be elucidated. Conflicting data exist with regard to the role of the tumor suppressor gene p53 in response of tumor cells to drugs. In theory loss of normal p53 by mutations may result both in enhanced and decreased sensitivity to DNA damaging drugs, because of its presumed roles in

cell cycle arrest and apoptosis. In 40-50% of ovarian carcinomas mutations in the p53 gene can be found. In human ovarian carcinoma positive p53 immunostaining (strongly related to the presence of mutations) is related to shorter survival, and parameters of more aggressive tumor growth, but not to worse response to chemotherapy. In conclusion it appears from this review, that although the role of genetic changes in ovarian carcinoma with regard to tumor growth characteristics and response to chemotherapy is far from elucidated, the identification of the diverse genetic changes offers opportunities for new approaches in the treatment of patients with ovarian carcinoma.

In **chapter 2** a review is given of the mechanisms of resistance to the four classes of drugs that are widely used in ovarian carcinoma: platinum (cisplatin/carboplatin) compounds, classical alkylating agents (cyclophosphamide/melphalan), natural drugs (doxorubicin), and "new drugs" (taxol and its synthetic analogue taxotere). As both platinum and classical alkylating agents mediate their cytotoxicity by the formation of drug-DNA adducts, encountered drug resistance mechanisms are (in part) comparable. In cultured ovarian carcinoma cells increased detoxification by binding of drugs to glutathione (possibly catalyzed by glutathione S-transferases) and increased repair of DNA damage have been identified as the most prominent resistance mechanisms to these drugs. Studies on the role of glutathione and DNA repair mechanisms in human ovarian carcinoma are hampered by intratumor heterogeneity (glutathione) and the complexity of enzyme systems involved in DNA repair. Resistance to doxorubicin appears to be mediated by enhanced efflux from the cell by increased expression of membrane glycoproteins acting as efflux pumps, such as P-glycoprotein. Resistance to doxorubicin can also be due to quantitative and/or qualitative changes in the nuclear target of doxorubicin, topoisomerase (Topo) II. Finally, resistance to taxol may be mediated by enhanced expression of P-glycoprotein, while presumed other mechanisms such as alterations in tubulin structure (cellular target of taxol), and changes in levels of tubulins are largely unresolved. This review shows that many cell biological factors may be involved in drug resistance. The relevance of the identification of most of these factors in ovarian carcinoma patients remains to be established. Several ways to modulate the reviewed resistance mechanisms are also described in this chapter.

In **chapter 3** P-glycoprotein expression and DNA topoisomerase I and II were studied in benign and malignant epithelial ovarian tumors. P-glycoprotein expression was analyzed immunohistochemically in cryostat sections of tumor specimens. In the same specimens topoisomerase I and II activity were measured. P-glycoprotein expression (range: 5-100% positive staining cells) was found in 3 of 6 benign ovarian cystadenomas, 0 of 2 borderline ovarian tumors, 15 of 21 untreated ovarian carci-

nomas, and 8 of 13 platinum/cyclophosphamide treated ovarian carcinomas. Median topoisomerase I and II activity were elevated in malignant ovarian tumors compared to benign and borderline tumors. No difference was found between median topoisomerase I activity in untreated ovarian carcinomas and platinum/cyclophosphamide treated ovarian carcinomas. High topoisomerase II activity ($\geq 8 \times 10^2$ U/mg protein) was more frequent in untreated compared to platinum/cyclophosphamide treated samples. Respectively, 8- and 16-fold differences in topoisomerase I and II activity were found in the malignant tumors. Topoisomerase II activity in malignant tumors correlated with topoisomerase I activity and the tumor volume index (percentage of tumor cells in the biopsy). However, this last relative weak correlation can not explain the 16-fold differences in topoisomerase II activity in malignant tumors. Mitotic index, and P-glycoprotein expression in the tumors did not correlate with topoisomerase I or II activity. An important variability in P-glycoprotein expression and topoisomerase I and II activity was observed in patients with ovarian carcinoma, which in part may be responsible for the varying response of ovarian carcinomas to cytotoxic drugs such as doxorubicin.

In **chapter 4** both quantitative and qualitative aspects of topoisomerase I and II were studied in 17 malignant ovarian tumors (8 untreated and 9 after platinum/cyclophosphamide chemotherapy). Median topoisomerase II activity was again lower in tumors after chemotherapy in comparison to untreated tumors, while no differences were found for topoisomerase I activity in tumors before and after chemotherapy, as was also described in chapter 3. It is presumed that cytotoxicity of topoisomerase I or II targeted drugs is mediated by the irreversible binding of topoisomerase I or II to DNA in the so-called "cleavable complex" by these drugs. In this study topoisomerase I and II, isolated from human malignant ovarian tumors, could be stimulated by camptothecin (topoisomerase I targeted drug) and teniposide (topoisomerase II targeted drug), respectively to induce cleavable complex formation. This cleavable complex formation had never been shown before in the literature for ovarian carcinoma or other malignancies. It shows that topoisomerases are real targets for chemotherapy in patients with ovarian carcinoma. In this study no indications for qualitative differences in topoisomerase II were found because: teniposide induced cleavable complex formation correlated with topoisomerase II activity; topoisomerase II decatenation activity was equally inhibited by teniposide in all tumors; and no differences were found in topoisomerase II cleavage sites patterns in plasmid DNA for all tumors. With regard to qualitative aspects of topoisomerase I it was shown that cleavable complex formation of topoisomerase I by camptothecin did not correlate with topoisomerase I activity, while topoisomerase I activity in all tumors could equally be inhibited by camptothecin. These results indicate that qualitative differences in topoisomerase I may exist, but further characterization is needed. By immunoblotting topoisomerase

IIα protein expression was detected in four of eight untreated tumors and three of nine tumors after chemotherapy, whereas in all tumors a degradation product of topoisomerase IIß was detected. Topoisomerase I protein was detected in all tumors at varying levels. From data in tumor cell lines it appears that the cleavable complex assay reflects both quantitative and qualitative changes in topoisomerase I and II. In combination with the feasibility of the cleavable complex assay for topoisomerase I and II in human malignant tumors, which was found in this study, it appears that at present the determination of cleavable complex formation by tumors is the most promising parameter of topoisomerase I or II expression in human tumors to be related to response to topoisomerase I or II targeted chemotherapy.

In **chapter 5** a detailed molecular analysis of the topoisomerase IIα gene and its expression was carried out. Topoisomerase IIα expression was determined by immunoblot analysis. In addition, the topoisomerase IIα gene was examined in order to discover whether the expression of topoisomerase IIα was influenced by gross genetic changes. We used methodology which allows the sequential extraction of protein and genomic DNA from the same biopsy. Thus, both gene expression and genetic analysis was carried out without the need to process separate pieces of tissue. This approach makes efficient use of small biopsy samples and importantly, allows data on gene expression to be correlated directly with genetic changes. Topoisomerase IIα protein expression was analyzed in 54 tumors. Topoisomerase IIα protein expression was detected in 65% of ovarian tumors with a 16- fold range in levels. In high grade and advanced-stage tumors topoisomerase IIα expression was higher than in low grade and early-stage tumors. Of 86 malignant ovarian tumors studied only one tumor showed amplification of c-erbB-2 and none had amplification of topoisomerase IIα sequences. Molecular analysis of the topoisomerase IIα locus failed to reveal any gross genetic alteration which could account for the variation in levels of expression.

In **chapter 6** glutathione S-transferase (GST) isozyme composition, isozyme quantities and enzymatic activity were investigated in benign ovarian tumors and malignant ovarian tumors, before and after chemotherapy. Enzymatic activity of GST in cytosols was measured by determining 1-chloro-2,4-dinitrobenzene conjugation with glutathione, and cytosolic GST subunits were determined by wide pore reversed phase high pressure liquid chromatography. Both GST activity and GST pi amount were not related to histopathologic type, differentiation grade, or tumor volume index in untreated malignant tumors. GST isozyme patterns were identical in benign tumors and malignant tumors before and after platinum/cyclophosphamide chemotherapy, while GST pi was the predominant transferase. Surprisingly, mean GST activity and GST pi amount were decreased in malignant ovarian tumors after platinum/cyclophosphamide chemotherapy compared to untreated ovarian malignant tumors. No

relation was found between GST pi amount and response to platinum/cyclophosphamide chemotherapy. Thus, within the limitations of this study no arguments were found for a role for GSTs in drug resistance of malignant ovarian tumors to platinum/cyclophosphamide chemotherapy.

In **chapter 7** the presence and prognostic value was studied of immunostaining of P-glycoprotein, GST pi, c-erbB-2 and p53 in patients with advanced-stage ovarian carcinoma with respect to response to chemotherapy and (progression free) survival. Immunostaining of P-glycoprotein, GST pi, c-erbB-2, and p53 was performed on paraffin-embedded sections of 89 primary tumors and 38 residual tumors after chemotherapy (P-glycoprotein and GST pi) in a well documented series of patients with advanced-stage ovarian carcinoma uniformly treated in first line with cisplatin, cyclophosphamide, and doxorubicin with long term follow-up. The results of immunostaining were related to clinicopathologic prognostic factors and (progression free) survival. P-glycoprotein and GST pi immunoreactivity were present in 13 cases (15%) and 79 cases (89%), respectively, and were not associated with any other clinicopathologic prognostic factor or (progression free) survival. C-erbB-2 immunoreactivity was present in 18 cases (20%), and was associated with undifferentiated histiotype, but not with (progression free) survival. P53 immunoreactivity was present in the nuclei of 31 cases (35%) and cytoplasm of 9 cases (10%). Nuclear p53 staining was associated with poorly differentiated tumors, presence of > 1 liter ascites and residual tumor after first laparotomy > 2 cm. The following (in order of significance) factors were associated with shorter progression free survival in log rank analysis: stage III or IV disease, no response to chemotherapy, nuclear p53 staining, presence of > 1 liter ascites, residual tumor after first laparotomy > 2 cm, and age > 50 years. Nuclear p53 staining was also associated with shorter overall survival. After adjustment for presence of > 1 liter ascites, and age > 50 years nuclear p53 staining did not retain independent prognostic significance in stage III/IV tumors. The frequency of P-glycoprotein staining in patients with residual tumors after chemotherapy (18/38 cases) was higher in comparison to untreated tumors (13/89 cases). No combination of immunohistochemical or clinicopathologic parameters was able to predict response to chemotherapy adequately. Based on these observations nuclear immunoreactivity of p53 in ovarian carcinomas is associated with shorter progression free survival and determinants of more aggressive tumor growth, but not with response to chemotherapy. Higher frequency of P-glycoprotein immunoreactivity in residual tumors after chemotherapy points to induction of P-glycoprotein in ovarian carcinomas by doxorubicin containing combination chemotherapy. The determination of P-glycoprotein, GST pi, c-erbB-2 and p53 does not permit more adequate prediction of response to chemotherapy.

In **chapter 8** a possible relation between interleukin (IL)-6 levels in cystic fluids of benign and malignant ovarian tumors and pretreatment serum C-reactive protein, platelet counts, and hemoglobin levels was evaluated. Bio-assay and enzyme-linked immunosorbent assay (ELISA) were used to determine the levels of IL-6 in cystic fluids and serum from patients with benign and malignant ovarian tumors. Median IL-6 was higher in cystic fluids of malignant tumors when compared with cystic fluids of benign tumors (both assays). Serum IL-6 levels in patients with malignant tumors were comparable to Il-6 levels in patients with benign tumors, while C-reactive protein levels were higher in patients with malignant tumors. Cystic fluid IL-6 levels were related to serum C-reactive protein levels (for both assays); platelet counts (both assays), and inversely related to hemoglobin levels (both assays). Interestingly, analogous high platelet counts and low hemoglobin also occur in patients after the administration of recombinant human IL-6 (1). It appears from this study that IL-6 levels are higher in cystic fluids of malignant ovarian tumors compared with benign tumors. The relation of high cystic fluid IL-6 levels with high serum C-reactive protein, high platelet counts, and low hemoglobin points to a possible causative role of tumor-derived IL-6 in the appearance of general side-effects in ovarian carcinoma, which have recently been recognized as prognostic factors (2).

In **chapter 9** the expression and prognostic value of LRP (a recently identified protein associated with the non-P-gp multidrug resistance phenotype) was evaluated in patients with advanced-stage ovarian carcinoma with respect to response to chemotherapy and (progression free) survival. Immunostaining of LRP and P-glycoprotein was performed on frozen sections of 66 stage III/IV primary tumors using monoclonal antibody LRP-56 and two P-glycoprotein-specific monoclonal antibodies, respectively. Fifty-seven patients received chemotherapy (platinum-based in 88%) after surgery. The results of immunostaining were related to clinicopathologic prognostic factors in ovarian carcinoma and (progression free) survival. LRP and P-glycoprotein immunoreactivity were present in respectively, 74% and 13% of cases. LRP expression tended to be negatively associated with high tumor grade. LRP, but not P-glycoprotein immunostaining was associated with complete response to induction chemotherapy (50% complete responses in tumors with negative LRP staining versus 8% complete responses in tumors with positive LRP staining). Patients with positive immunostaining for LRP in the tumor had a shorter progression free and overall survival in comparison to patients with negative immunostaining. P-glycoprotein immunostaining at diagnosis had no prognostic value. These results indicate that LRP, but not P-glycoprotein, defines a drug resistant phenotype expressed intrinsically in this group of patients with ovarian carcinoma. LRP has strong independent negative prognostic significance with regard to survival.

In this thesis diverse cell biological features related to drug resistance and/or tumor aggressiveness have been examined. Our studies on topoisomerase I and II show that these nuclear enzymes are real targets for cytotoxic drugs in ovarian carcinoma. The cleavable complex assay appears to be the most promising parameter of topoisomerase I and II expression in malignant tumors to be related to clinical response to topoisomerase I or II targeted chemotherapy. It will be interesting in future studies to relate the cleavable complex formation by tumor extracted topoisomerase I to the response to topoisomerase I targeted drugs, such as topotecan, that is currently used as monotherapy in phase II trials in patients with ovarian carcinoma.

Our studies and those by others on P-glycoprotein show that the reported frequency of P-glycoprotein expression is dependent on the methodology used to determine P-glycoprotein expression. Overall P-glycoprotein expression in primary ovarian carcinomas seems to be low, but can be induced by natural drugs such as doxorubicin. The introduction of the "new" natural product taxol in first and second line chemotherapy in patients with ovarian carcinoma may enhance the significance of resistance mediated by P-glycoprotein. Therefore, compounds that modulate P-glycoprotein mediated resistance may be useful to combine with drugs such as taxol.

From in vitro studies it appears that detoxification of drugs by direct binding or conjugation to the cytoplasmatic non-protein glutathione is one of the more prominent cellular defense mechanisms with regard to resistance to platinum compounds and alkylating agents. Our studies show no prognostic value for the GST pi isozyme with regard to response to chemotherapy and survival in primary ovarian carcinomas. In tumors after platinum-based chemotherapy no induction of GST pi or other GST isozymes was observed. Therefore, GST pi is not an adequate parameter of the detoxification capacity of ovarian carcinomas. Glutathione levels in the tumor itself appear to be more promising parameters of cellular detoxification capacity to relate to response to platinum-based chemotherapy. However, technical limitations in glutathione determination and, more importantly, large intratumor heterogeneity of glutathione levels in ovarian carcinomas prohibit such studies. Perhaps the determination of the expression of rate-limiting glutathione synthesis enzymes, such as gamma-glutamyl transpeptidase and gamma-glutamylcysteine synthetase are better parameters of glutathione-related cellular detoxification capacity. It will be interesting to relate the expression of these enzymes in primary ovarian carcinomas to response to platinum-based chemotherapy. At the same time the results have to be awaited of clinical studies in patients with ovarian carcinoma, testing the efficacy of depleting glutathione levels by buthionine sulfoximine as a means of overcoming drug resistance to alkylating agents such as melphalan.

Our study on LRP shows a surprisingly strong relation between LRP expression and response to platinum-based chemotherapy. More information is needed on how LRP expression confers resistance to platinum-based chemotherapy. However, if the

strong relation of LRP expression and response to chemotherapy is confirmed in future prospective studies, it will be important to investigate which compounds are able to modulate LRP mediated drug resistance. The combination of platinum-based chemotherapy with such compounds may have additive antitumor activity in patients with drug resistant tumors.

Our study on IL-6 in serum and cystic fluids of patients with benign and malignant tumors points to a causative role of IL-6 in the appearance of general side-effects in ovarian carcinoma patients. In vivo and in vitro data in other diseases have established the role of IL-6 in the appearance of general side-effects such as thrombocytosis, anemia, fever, and weight loss. Apart from a potential role in the generation of general side-effects in ovarian carcinoma patients it remains unclear if IL-6 has a role as (autocrine or paracrine) growth promoting factor in ovarian tumor cells. Wild type p53 has been reported to repress the transcription of IL-6, which ability is lost by mutant p53 (3). Currently, we are performing in vitro and in vivo studies on the relation between p53 expression and IL-6 expression and/or production by malignant ovarian tumor cells.

The identification of the diverse genetic changes involved in the development of ovarian carcinomas offers opportunities for new approaches in the treatment of patients with ovarian carcinoma. Positive immunostaining of the tumor suppressor gene p53 (pointing to the presence of p53 mutations) appears to be related to parameters of more aggressive tumor growth and shorter survival in advanced-stage ovarian carcinomas. For tumor suppressor genes where mutations result in loss of function, the goal of gene therapy is to reintroduce the lost wild-type gene (4). In vitro studies showed that p53 defective (lung, bladder, and colon) cell lines transfected with a retroviral vector containing the normal p53 gene had reduced growth as compared to control cells (5). The difficulty in this approach in the clinic lies in delivering actively expressed vectors to every single tumor cell. The fact that metastases of ovarian carcinoma are almost exclusively limited to the intra-abdominal cavity may be an advantage when considering this kind of therapy in advanced-stage ovarian carcinoma patients. It remains to be elucidated how reintroduction of normal p53 into p53 defective cells (by mutations) will influence the sensitivity of tumor cells to the various cytotoxic drugs (6). Downregulation of abnormal oncogene expression has been shown to revert the malignant phenotype in various tumor cell lines (3). The (also in our study) reported overexpression of the c-erbB-2 oncogene in 20-30% of patients with ovarian carcinoma presents this oncogene as another possible target for gene therapy. Inhibiting oncogenic protein production by administering "antigenes" should lead to restoration of normal growth control (4). Combining cytotoxic drugs with monoclonal antibodies against c-erbB-2 is another example of new approaches in the treatment of patients with ovarian carcinoma, that in experimental studies appear to result in more efficient anti-tumor activity.

After the introduction of platinum-based drugs into combination chemotherapy the last two decades brought no important changes in chemotherapy for patients with advanced-stage ovarian carcinoma. The recent (intended) inclusion of taxol (and taxotere) in first and second line chemotherapy may improve the poor prognosis for patients with advanced-stage ovarian carcinoma. However, especially the exponentially developing understanding of underlying (genetic) mechanisms of dysregulated tumor growth and drug resistance will result in a new arsenal of drugs with specific anti-tumor activity. Therefore, hopefully more changes in the treatment of advanced-stage ovarian carcinomas are to be awaited in the next two decades.

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Chapter 11

Summary in Dutch (Samenvatting)

Het epitheliale ovariumcarcinoom kent de hoogste mortaliteit van alle gynaecologische maligniteiten. De oorzaak hiervan is, dat bij het merendeel van de patiëntes met een ovariumcarcinoom de ziekte pas in een vergevorderd stadium wordt ontdekt. Radicaal chirurgische verwijdering van de tumor is daardoor vaak niet meer mogelijk. Eventuele genezing van deze patiëntes zal slechts met een systemische behandeling in de vorm van chemotherapie bereikt kunnen worden, nadat door middel van operatie zo veel mogelijk tumor is verwijderd. Helaas is de effectiviteit van de momenteel ter beschikking staande chemotherapie onvoldoende. Hoewel in eerste instantie vaak een goede reaktie van het ovariumcarcinoom op chemotherapie wordt gezien, is de duur in het algemeen kort. De reaktie op chemotherapie van patiëntes bij wie na afronding van de eerstelijns chemotherapie nog tumor aanwezig is, of bij wie de ziekte zich opnieuw manifesteert, is in het algemeen slecht. Dit leidt uiteindelijk tot sombere overlevingscijfers (5-jaars overleving: 10-20%). Deze klinische gegevens maken duidelijk, dat intrinsieke en verworven resistentie (ongevoeligheid) van tumorcellen tegen chemotherapie een belangrijk probleem vormen in de behandeling van patiëntes met een gemetastaseerd ovariumcarcinoom.

Bekende prognostische factoren in het ovariumcarcinoom zijn: leeftijd, algemene conditie, stadium van de tumor, de hoeveelheid resttumor na de eerste operatie, aan- of afwezigheid van ascites, differentiatiegraad van de tumor, histopathologisch type en DNA ploïdie. Deze prognostische factoren zijn niet gerelateerd aan de gevoeligheid van de tumor voor chemotherapie. Momenteel zijn er geen prognostische factoren bekend, die het mogelijk maken de reactie van een tumor op chemotherapie te voorspellen. Om de effectiviteit van chemotherapie te verbeteren, is inzicht nodig in celbiologische factoren die betrokken zijn bij de aanwezigheid of het ontstaan van resistentie tegen chemotherapie.

Onderzoek in (ovarium)tumorcellijnen heeft meer inzicht gegeven in cellulaire mechanismen die betrokken kunnen zijn bij resistentie tegen chemotherapie. Deze mechanismen kunnen op verschillende cellulaire niveaus worden gevonden: 1. *de celmembraan:* celmembraan gebonden glycoproteïnes, zoals P-glycoproteïne (P-gp), zijn in staat om verschillende cytostatica zoals epipodophyllotoxines, vincristine, doxorubicine en taxol uit de cel te pompen. Hierdoor wordt de intracellulaire concentratie van het betrokken cytostaticum verlaagd. Een verhoogd voorkomen van het P-gp in de celmembraan resulteert in resistentie tegen verschillende cytostatica (de zogenaamde multidrug resistentie (MDR)); 2: *het cytoplasma:* cytostatica, zoals

platinum bevattende en alkylerende middelen, kunnen direct of door conjugatie aan het cytoplasmatische glutathion gebonden worden. Op deze wijze wordt voorkomen, dat deze cytostatica hun doel, namelijk het DNA in de celkern, bereiken. Cytoplasmatische enzymen, zoals glutathion S-transferases (GSTs) kunnen de binding van cytostatica aan glutathion bevorderen. Een verhoogd voorkomen van cytoplasmatisch glutathion of GSTs kan leiden tot een versterkte cellulaire detoxificatie van cytostatica; 3: *de celkern:* a: topoisomerase (Topo) I en II zijn kernenzymen die betrokken zijn bij transcriptie en replicatie van DNA. Tegelijkertijd zijn Topo I en II aangrijpingspunten voor verschillende cytostatica, zoals Topo I voor camptothecine en Topo II voor doxorubicine. Resistentie tegen deze cytostatica kan ontstaan, doordat tumorcellen de hoeveelheid Topo I of II verlagen, waardoor er minder aangrijpingspunt voor het cytostaticum aanwezig is, of doordat tumorcellen de eiwitconfiguratie van Topo I of II zodanig veranderen, dat de cytostatica niet meer reaktief kunnen zijn met Topo I of II; b: in de celkern zijn complexe enzymsystemen aanwezig die verantwoordelijk zijn voor herstel van DNA schade. Cytostatica, zoals platinum bevattende en alkylerende middelen, oefenen hun werking uit door het (onherstelbaar) beschadigen van het DNA. Resistentie tegen deze cytostatica kan onder andere worden veroorzaakt door een versnelde reparatie van DNA schade door verhoogde expressie van DNA herstel enzymen.

Het in experimentele studies verkregen inzicht in oorzaken van resistentie tegen cytostatica dient vertaald te worden naar de gecompliceerde werkelijkheid van de patiënte met een ovariumcarcinoom. De identificatie in een maligne tumor van specifieke celbiologische parameters, die betrokken zijn bij resistentie tegen cytostatica dient te leiden tot een meer rationele, eventueel geïndividualiseerde keuze van cytostatica, of tot de toepassing van middelen, die specifiek een aangetoond resistentiemechanisme tegengaan.

In dit proefschrift worden in het humane ovariumcarcinoom verschillende celbiologische factoren bestudeerd waarvan in het laboratorium is aangetoond, dat zij betrokken kunnen zijn bij resistentie tegen cytostatica of bij agressieve tumorgroei, hetgeen twee op zichzelf staande eigenschappen van het biologische gedrag van een tumor zijn.

Meerdere achtereenvolgende genetische veranderingen treden op in de ontwikkeling van een ovariumcarcinoom. Een ovariumcarcinoom bevat dan ook een reservoir van genetisch heterogene tumorcellen met verschillende eigenschappen voor wat betreft groei, differentiatie, metastasering en resistentie tegen cytostatica. In **hoofdstuk 1** wordt een literatuuroverzicht gegeven van in het ovariumcarcinoom beschreven genetische veranderingen in mogelijke relatie tot resistentie tegen cytostatica.

In maligne tumorcellen treedt ongeveer één spontane mutatie op per 10^4-10^6

celdelingen. De kans op het optreden van een aan resistentie tegen cytostatica gerelateerde mutatie is derhalve evenredig met het volume van een maligne tumor. De consequentie hiervan is, dat bij chirurgie voor een primair ovariumcarcinoom getracht moet worden zoveel mogelijk tumor te verwijderen.

Cytogenetische studies en studies, waarbij met behulp van moleculair biologische technieken verlies van chromosomale heterozygositeit wordt bepaald, worden gebruikt om het verlies van delen van chromosomen en tevens translocaties op te sporen. Wanneer verlies van specifieke chromosoomdelen met hoge frequentie wordt waargenomen in verschillende (ovarium) carcinomen, duidt dit op de mogelijke aanwezigheid van een gen op dit deel van het chromosoom, dat betrokken is bij het ontstaan of de verdere ontwikkeling van het ovariumcarcinoom. In het ovariumcarcinoom is een hoge frequentie waargenomen van verlies van de chromosoomarmen 6p, 17p, en 17q. Er zijn in het ovariumcarcinoom geen gegevens beschikbaar over een mogelijke relatie van specifieke numerieke genetische veranderingen met resistentie tegen cytostatica.

Overexpressie van het c-erbB-2 oncogen en expressie van de epidermale groeifactor receptor (EGF-R) worden in het ovariumcarcinoom waargenomen in respectievelijk 20-30% en 50-60%. De (over)expressie van deze transmembrane glycoproteïnes lijkt op zichzelf geen negatieve prognostische betekenis te hebben, maar meerdere studies tonen aan dat (over)expressie van c-erbB-2 of EGF-R wel een rol kan spelen in de groeiregulatie van een belangrijk deel van de ovariumcarcinomen. Interessant is dan ook dat in experimentele studies het blokkeren van deze groeifactor receptoren door middel van monoclonale antilichamen tegen deze receptoren resulteert in groeivertraging. Wanneer deze monoclonale antilichamen echter worden gegeven in combinatie met een cytostaticum, zoals cisplatinum, treedt een beduidend sterker cytotoxische effect op in vergelijking met de werking van cisplatinum alleen. Wellicht kunnen in de toekomst dergelijke monoclonale antilichamen in de kliniek een toepassing vinden.

De expressie van de oncogenen, c-fos, c-jun en c-myc kan toenemen, doordat tumorcellen aan bepaalde (DNA beschadigende) cytostatica worden blootgesteld. Verhoogde expressie van deze nucleaire transcriptie-factoren resulteert in hogere niveaus van kernenzymen, die betrokken zijn bij het herstel van beschadigd DNA, hetgeen uiteindelijk leidt tot resistentie. Of de expressie van de c-fos, c-jun en c-myc oncogenen inderdaad van belang is voor het biologisch gedrag (in casu de gevoeligheid voor cytostatica) van ovariumcarcinomen is momenteel niet voldoende onderzocht.

Experimentele studies laten met elkaar strijdige resultaten zien betreffende de rol van het tumorsuppressor-gen p53 in relatie tot de reaktie van tumorcellen op cytostatica. Enerzijds is waargenomen dat het blootstellen van tumorcellen aan DNA beschadigende cytostatica leidt tot een verhoogde expressie van normaal p53. Hierdoor

treedt een verlenging op van de G_0/G_1 fase van de celcyclus, waardoor de cel gelegenheid krijgt om aangerichte DNA schade te repareren. Op deze manier leidt het verlies van normaal p53 (bijvoorbeeld door een mutatie) tot een grotere gevoeligheid voor cytostatica, omdat het vermogen tot reparatie van DNA schade verminderd is. Anderzijds is echter waargenomen, dat normaal p53 essentieel is voor een cel om in apoptose (geprogrammeerde celdood) te gaan na het optreden van DNA schade. Bij het verlies van normaal p53 door een mutatie verliest een cel het vermogen om in apoptose te gaan en kan daardoor ongevoeliger worden voor cytostatica. In 40-50% van de gemetastaseerde humane ovariumcarcinomen kunnen p53 mutaties worden aangetoond die resulteren in het verlies van de normale p53 functie. In humane ovariumcarcinomen is de aanwezigheid van p53 mutaties sterk geassocieerd met kortere overleving en parameters van agressievere tumorgroei, maar niet met reaktie op cytostatica.

Concluderend kan gesteld worden, dat hoewel de rol van de diverse genetische veranderingen in het ovariumcarcinoom met betrekking tot tumorgroei en resistentie tegen cytostatica verre van opgehelderd is, het aantonen van verschillende genetische veranderingen wellicht mogelijkheden biedt voor nieuwe vormen van (chemo)-therapie in het ovariumcarcinoom.

In **hoofdstuk 2** wordt een overzicht van de literatuur gegeven betreffende de verschillende mechanismen van resistentie tegen cytostatica, die het meest gebruikt worden voor de behandeling van patiëntes met ovariumcarcinoom, namelijk: platinum (cisplatinum/carboplatin) bevattende cytostatica, alkylerende middelen (cyclofosfamide/melfalan), natuurlijke producten (doxorubicine) en "nieuwe" middelen (taxol/taxotere). Zowel platinum bevattende als klassieke alkylerende middelen oefenen hun cytostatische werking uit door de formatie van cytostaticum-DNA adducten. Als gevolg hiervan is er een gedeeltelijke overlap in de beschreven resistentiemechanismen. In ovariumcarcinoom-cellijnen blijken versterkte detoxificatie (door binding van cytostatica aan glutathion) en versneld herstel van door cytostatica aangerichte DNA schade de belangrijkste resistentiemechanismen te zijn tegen platinum bevattende en klassieke alkylerende middelen. Studies in biopsieën van humane ovariumtumoren naar de klinische relevantie van deze twee resistentiemechanismen worden enerzijds belemmerd door technische problemen (glutathion), anderzijds door de complexiteit van het betrokken mechanisme (versterkt herstel van DNA schade). Resistentie tegen doxorubicine in ovariumcarcinoom-cellijnen kan veroorzaakt worden door een verhoogde expressie van het eerder beschreven P-gp, die leidt tot een verlaging van de intracellulaire concentratie van doxorubicine. Resistentie tegen doxorubicine kan eveneens optreden door kwantitatieve en/of kwalitatieve veranderingen in Topo II, het aangrijpingspunt in de kern voor doxorubicine. Ook resistentie tegen taxol tenslotte, kan veroorzaakt worden door verhoogde expressie van P-gp.

Andere resistentiemechanismen tegen taxol, zoals veranderingen in de cellulaire tubuline structuren (aangrijpingspunt van taxol) en wisselende niveaus van tubulines, zijn grotendeels nog onvoldoende onderzocht. Dit literatuuroverzicht laat zien dat meerdere celbiologische factoren betrokken kunnen zijn bij resistentie tegen cytostatica. De klinische relevantie van het bepalen van het merendeel van deze factoren in tumoren van patiëntes met een ovariumcarcinoom dient nog aangetoond te worden. Verschillende manieren om de genoemde resistentiemechanismen te moduleren worden eveneens beschreven in dit hoofdstuk.

In tumorcellijnen blijken expressie van P-gp en Topo I en II activiteit belangrijke parameters te zijn voor resistentie tegen cytostatica zoals camptothecine en doxorubicine. In **hoofdstuk 3** worden de expressie van P-gp en Topo I en II bestudeerd in benigne en maligne epitheliale ovariumtumoren. P-gp expressie werd bepaald door middel van immunohistochemie in vriescoupes van tumorbiopsieën. In dezelfde biopsieën werd ook Topo I en II activiteit gemeten. P-gp expressie (spreiding: 5-100% aankleurende cellen) werd gevonden in 3 van 6 benigne cystadenomen van het ovarium, geen van 2 borderline ovariumtumoren, 15 van 21 primaire ovariumcarcinomen en 8 van 13 ovariumcarcinomen verkregen na behandeling met cytostatica. De mediane Topo I en II activiteit was hoger in de primaire maligne tumoren in vergelijking met de benigne en borderline tumoren. De mediane Topo I activiteit was gelijk in ovariumcarcinomen verkregen voor en na behandeling met cytostatica. Hoge Topo II activiteit werd frequenter waargenomen in primaire ovariumcarcinomen dan in carcinomen verkregen na behandeling met cytostatica. Respectievelijk 8- en 16-voudige verschillen werden waargenomen in Topo I en II activiteit in de verschillende maligne tumoren. Topo II activiteit in de maligne tumoren correleerde zwak met Topo I activiteit en met de tumor volume index (percentage maligne cellen in biopsie). Deze laatste (zwakke) correlatie verklaart echter niet de 8- en 16-voudige verschillen in Topo I en II activiteit in ovariumcarcinomen. Mitotische index en P-gp expressie correleerden niet met Topo I of II activiteit. Deze studie laat zien, dat maligne ovariumtumoren onderling sterk verschillen in P-gp expressie en Topo I en II activiteit. Wellicht zijn deze onderlinge verschillen in P-gp expressie en Topo II activiteit (gedeeltelijk) verantwoordelijk voor de wisselende reaktie van ovariumcarcinomen op cytostatica zoals doxorubicine.

In **hoofdstuk 4** worden zowel kwantitatieve als kwalitatieve aspecten van Topo I en II onderzocht in 8 primaire ovariumcarcinomen en 9 ovariumcarcinomen verkregen na behandeling met cytostatica. De mediane Topo II activiteit was, evenals in hoofdstuk 3, lager in ovariumcarcinomen verkregen na behandeling met cytostatica in vergelijking met primaire ovariumcarcinomen. In Topo I activiteit werden dergelijke verschillen niet waargenomen. In studies in tumorcellijnen is aangetoond dat het cyto-

toxische effect van cytostatica die op Topo I of II aangrijpen, veroorzaakt wordt doordat deze cytostatica de normaliter passagère binding van Topo I en II aan het DNA irreversibel maken, waarbij een zogenaamd cleavable complex wordt gevormd. Deze studie in ovariumcarcinomen laat zien dat uit humane tumoren geïsoleerd Topo I en II inderdaad tot cleavable complex formatie in staat zijn in de aanwezigheid van Topo I of II aangrijpende cytostatica. Dergelijke cleavable complex formatie was nog niet eerder aangetoond in humane (ovarium) tumoren en het wijst erop dat Topo I en II reële aangrijpingspunten zijn voor cytostatica in patiëntes met een ovariumcarcinoom. Geen aanwijzingen werden gevonden voor kwalitatieve verschillen in Topo II, terwijl de resultaten voor Topo I wel in deze richting wezen. Van het Topo II eiwit zijn twee isovormen beschreven, het Topo IIα en Topo IIß. Deze twee isovormen zijn verschillend voor wat betreft gevoeligheid voor de diverse Topo II aangrijpende cytostatica. Derhalve kunnen zowel het absolute niveau als ook de onderlinge ratio van de twee isovormen van Topo II de gevoeligheid van tumorcellen voor cytostatica bepalen. Door middel van immunoblotting werd in 4 van de 8 primaire ovariumcarcinomen Topo IIα eiwit aangetoond en in 3 van de 9 ovariumcarcinomen die waren verkregen na behandeling met cytostatica. In alle ovariumcarcinomen werd een afbraakproduct van Topo IIß aangetoond. Topo I eiwit werd eveneens in alle ovariumcarcinomen aangetoond. Het feit dat cleavable complex formatie eenvoudig te meten was in alle ovariumcarcinomen, gecombineerd met de waarnemingen in tumorcellijnen, dat het meten van cleavable complex formatie zowel kwantitatieve als kwalitatieve veranderingen in Topo I en II reflecteert, leidt tot de conclusie van dit hoofdstuk, dat momenteel het meten van cleavable complex formatie in humane maligniteiten de meest geschikte parameter is om te correleren met reaktie op Topo I of II aangrijpende cytostatica.

Het Topo IIα gen en het c-erbB-2 oncogen zijn beide gelegen op chromosoom 17q21-22. Onlangs is aangetoond in maligne mammatumoren, dat het Topo IIα gen geco-amplificeerd kan zijn met het in de directe nabijheid gelegen c-erbB-2 oncogen. Deze co-amplificatie kan een versterkte expressie van het Topo IIα eiwit tot gevolg hebben. Uit de literatuur komt naar voren, dat in ongeveer 20-30% van de epitheliale ovariumcarcinomen c-erbB-2 amplificatie wordt gevonden. Tevens wordt frequent ter plaatse van chromosoom 17q21-22 verlies van heterozygositeit waargenomen in het ovariumcarcinoom. In **hoofdstuk 5** wordt een gedetailleerde analyse van het Topo IIα gen beschreven in relatie tot Topo IIα eiwit niveaus. Het topo IIα eiwit niveau werd bepaald door middel van immunoblotting. In deze studie werd een methode toegepast die het mogelijk maakte kerneiwitten en DNA achtereenvolgens te isoleren uit hetzelfde biopt. Op deze wijze konden de bepaling van eiwit niveau en de genetische analyse verricht worden in één en hetzelfde tumorbiopt. Topo IIα eiwit werd aangetoond in 65% van de 54 ovariumcarcinomen met een 16-voudige spreiding in niveaus.

In slecht gedifferentieerde en gemetastaseerde ovariumcarcinomen werd een hoger Topo IIα eiwit niveau gevonden dan in goed gedifferentieerde en niet gemetastaseerde tumoren. In slechts één van in totaal 86 ovariumcarcinomen werd c-erbB-2 gen-amplificatie waargenomen zonder co-amplificatie van het Topo IIα gen. Moleculair genetische analyse van het Topo IIα gen toonde geen aanwijzingen voor grote genetische veranderingen die verantwoordelijk zouden kunnen zijn voor de gevonden variatie in Topo IIα eiwit niveaus.

Zoals reeds eerder gesteld, kunnen cytoplasmatische enzymen, zoals GSTs, de binding van cytostatica aan glutathion bevorderen. Een verhoogd voorkomen van glutathion of GSTs kan leiden tot een versterkte cellulaire detoxificatie van cytostatica. In **hoofdstuk 6** worden de verschillende isovormen van GST en de totale enzymatische GST activiteit onderzocht in benigne ovariumtumoren, primaire ovariumcarcinomen en ovariumcarcinomen verkregen na chemotherapie. De totale enzymatische aktiviteit van alle GSTs werd gemeten door middel van het bepalen van de conjugatie van 1-chloro-2,4-dinitrobenzene met glutathion, terwijl de verschillende isovormen van GST bepaald werden met behulp van high pressure liquid chromatografie (HPLC). GST isovorm patronen waren gelijk in benigne tumoren en maligne tumoren voor en na chemotherapie. GST pi was veruit de belangrijkste GST isovorm. Min of meer onverwacht werd gevonden, dat de totale GST activiteit en de hoeveelheid GST pi in ovariumcarcinomen verkregen na chemotherapie lager waren dan in primaire carcinomen. Er was derhalve geen aanwijzing voor inductie van GSTs door voorafgaande chemotherapie. Evenmin werd een relatie gevonden tussen het GST pi niveau in de primaire ovariumcarcinomen en de reaktie op chemotherapie. Rekening houdend met de beperkingen van deze studie kan de conclusie worden getrokken dat GSTs geen belangrijke rol spelen in resistentie van ovariumcarcinomen tegen chemotherapie.

In **hoofdstuk 7** worden het voorkomen en de prognostische waarde bestudeerd van P-gp, GST pi, c-erbB-2 en p53 in relatie tot de reaktie op chemotherapie en de overleving van 89 patiëntes met een gemetastaseerd ovariumcarcinoom. Door middel van immunohistochemie op paraffine coupes werden deze parameters bepaald in primaire tumoren en tevens in 38 tumoren van patiëntes, bij wie bij herbeoordelingslaparotomie nog resttumor werd aangetroffen. De bestudeerde patiëntengroep werd gekenmerkt door een uniforme samenstelling, uniforme eerste lijns chemotherapie (cisplatinum, doxorubicine, en cyclofosfamide) en goed gedocumenteerde en langdurige follow-up. De resultaten van de immunohistochemie werden gerelateerd aan klinische en pathologische prognostische factoren en overleving. P-gp en GST pi aankleuring werd gevonden in respectievelijk 13 (15%) en 79 (89%) primaire tumoren, waarbij

geen relatie werd gevonden met andere prognostische factoren, reaktie op chemotherapie of overleving. C-erbB-2 aankleuring werd gevonden in 18 (20%) primaire tumoren en was geassocieerd met ongedifferentieerde tumoren, maar niet met reaktie op chemotherapie of overleving. P53 aankleuring werd gevonden in de kern van 31 (35%) en in het cytoplasma van 9 (10%) primaire tumoren. P53 aankleuring in de kern was geassocieerd met slecht gedifferentieerde tumoren, de aanwezigheid van meer dan 1 liter ascites en met tumorresten na eerste operatie groter dan 2 cm, maar niet met reaktie op chemotherapie. De volgende mogelijke prognostische factoren waren in volgorde van significantie geassocieerd met een kortere overleving: hoger stadium ovariumcarcinoom, ontbreken van reaktie op chemotherapie, kernaankleuring van p53, aanwezigheid van meer dan 1 liter ascites en leeftijd boven 50 jaar. Na correctie voor de aanwezigheid van meer dan 1 liter ascites of leeftijd boven 50 jaar bleek, dat kernaankleuring van p53 geen onafhankelijke prognostische waarde heeft met betrekking tot overleving. P-gp aankleuring kwam vaker voor in tumoren verkregen na chemotherapie (18/38 tumoren) dan in primaire tumoren. De bepaling van P-gp, GST pi, c-erbB-2 en p53 droeg niet bij aan het voorspellen van de reaktie op chemotherapie. Op basis van deze gegevens kan gekonkludeerd worden, dat in het ovariumcarcinoom p53 kernaankleuring geassocieerd is met agressieve tumorgroei en slechtere overleving, maar niet met de reaktie op chemotherapie. Hogere P-gp aankleuring in tumoren na chemotherapie wijst op mogelijke inductie van P-gp niveau door voorafgaande chemotherapie.

Onlangs is gesuggereerd dat bij patiëntes met een ovariumcarcinoom een hoog aantal bloedplaatjes en een laag hemoglobine gehalte geassocieerd zijn met een kortere overleving (1). Interleukine-6 (IL-6) is een groeifactor met verschillende functies. Verhoogde expressie van IL-6 kan resulteren in verhoogde acute fase eiwitten in het bloed, zoals C-reactive protein (CRP), een verhoogd aantal bloedplaatjes en een verlaagd hemoglobine gehalte. Sommige epitheliale ovariumcarcinoom-cellijnen blijken IL-6 te produceren en tevens zijn verhoogde IL-6 waardes gevonden in bloed en ascites van patiëntes met een ovariumcarcinoom. Behandeling van patiënten met recombinant humaan IL-6 in het kader van ondersteunende therapie bij chemotherapie resulteert in hoge aantallen bloedplaatjes, maar ook in een laag hemoglobine gehalte (2). In **hoofdstuk 8** wordt een mogelijke relatie onderzocht tussen IL-6 concentraties in cystevocht en serum van patiëntes met benigne en maligne ovariumtumoren en CRP, aantal bloedplaatjes en hemoglobine gehalte in bloed. IL-6 waardes werden bepaald door middel van bio-assay en enzyme-linked immunosorbent assay (ELISA). Mediane IL-6 concentraties waren hoger in cystevocht van maligne tumoren dan van benigne tumoren. IL-6 concentraties in het serum waren vergelijkbaar in patiëntes met benigne en maligne tumoren, terwijl de CRP concentraties hoger waren in patiëntes met een maligne tumor. Hoge IL-6 concentraties in cyste-

vocht waren geassocieerd met hoge CRP concentraties, een hoog aantal bloedplaatjes en een laag hemoglobine gehalte. De in deze studie gevonden relatie van hoog IL-6 in cystevocht van ovariumtumoren met een hoog aantal bloedplaatjes en een laag hemoglobine gehalte wijst op een mogelijke causale rol van door ovariumtumorcellen aangemaakt IL-6 in het ontstaan van algemene ziekteverschijnselen bij patiëntes met een ovariumcarcinoom.

Het lung resistance protein (LRP) is recent beschreven door de groep van Scheper et al (3). Dit eiwit komt tot overexpressie in tumorcellijnen, waarin geen verhoogde expressie van P-gp is aangetoond, terwijl deze cellijnen wel resistent zijn voor verschillende cytostatica, zoals doxorubicine. In **hoofdstuk 9** worden de expressie van LRP en P-gp en de mogelijke relatie van LRP en P-gp expressie met reaktie op chemotherapie en overleving in patiëntes met een ovariumcarcinoom onderzocht. LRP en P-gp expressie werden met behulp van immunohistochemie bepaald in vriescoupes van 66 patiëntes met een primair ovariumcarcinoom, stadium III of IV. De resultaten van de immunohistochemie werden gerelateerd aan bekende prognostische factoren voor het ovariumcarcinoom en aan overleving. LRP en P-gp aankleuring werd gevonden in respectievelijk 49/66 (74%) en 9/66 (13%) van de primaire tumoren. Negatieve LRP aankleuring was in geringe mate geassocieerd met ongedifferentieerde tumoren en sterk geassocieerd met complete reaktie op eerste lijns chemotherapie (50% complete reaktie in LRP negatieve tumoren versus 8% complete reaktie in LRP positieve tumoren). Ook was de overlevingsduur korter voor de patiëntes met LRP positieve tumoren. Voor P-gp aankleuring werd geen relatie met reaktie op chemotherapie gevonden, noch een relatie met overleving. Deze studie laat zien, dat positieve LRP aankleuring in primaire ovariumcarcinomen wijst in de richting van intrinsieke resistentie tegen (diverse soorten) chemotherapie.

In dit proefschrift worden meerdere celbiologische factoren beschreven en onderzocht in het ovariumcarcinoom, die gerelateerd kunnen zijn aan resistentie tegen chemotherapie of agressieve tumorgroei. Onze studies naar Topo I en II in het ovariumcarcinoom laten zien, dat deze kernenzymen in verschillende mate tot expressie komen in ovariumcarcinomen en daadwerkelijke aangrijpingspunten zijn voor chemotherapie. Het bepalen van cleavable complex formatie in humane maligniteiten lijkt vooralsnog de meest belovende parameter van Topo I of II expressie te zijn om te relateren aan reaktie op Topo I of II aangrijpende chemotherapie. Het lijkt met name interessant om in toekomstige studies cleavable complex formatie door tumoren te relateren aan reaktie op Topo I aangrijpende cytostatica, zoals topotecan, dat momenteel in fase 2 studies als monotherapie wordt gebruikt in de tweedelijns behandeling van patiëntes met een ovariumcarcinoom.

Uit onze eigen studies en die van anderen blijkt, dat de frequentie van P-gp expressie in het ovariumcarcinoom sterk afhankelijk is van de methodologie die wordt gebruikt voor het bepalen van P-gp expressie. In het algemeen echter lijkt de expressie van P-gp in primaire ovariumcarcinomen laag te zijn. De expressie van P-gp lijkt wel te kunnen worden geïnduceerd door voorafgaande (doxorubicine bevattende) cytostatica. Het toenemende gebruik van taxol (en eventueel taxotère) in de eerste en tweede lijns behandeling van het ovariumcarcinoom doet het belang van P-gp gerelateerde resistentie tegen cytostatica waarschijnlijk toenemen. Wellicht kunnen in de toekomst daardoor middelen die in staat zijn P-gp gerelateerde resistentie op te heffen waardevol zijn in de behandeling van patiëntes met een ovariumcarcinoom.

Studies in ovariumcarcinoom-cellijnen die resistent zijn tegen platinum bevattende of alkylerende middelen laten zien, dat versterkte detoxificatie door een verhoogd cytoplasmatisch glutathion een van de meest prominente cellulaire resistentiemechanismen is. Onze studies naar GST activiteit en GST pi expressie laten zien dat deze cytoplasmatische enzymen, die de binding van cytostatica aan glutathion kunnen bevorderen, geen goede parameters zijn voor de detoxificatiecapaciteit van een tumor. Waarschijnlijk is het bepalen van het glutathion-gehalte in de tumor een betere maat hiervoor. Deze bepaling wordt echter bemoeilijkt door technische problemen en vooral ook door grote intratumor-variabiliteit van glutathion-gehaltes. Wellicht is de bepaling van de gehaltes van enzymen, die betrokken zijn bij de synthese van glutathion, zoals gamma-glutamyl transpeptidase en gamma-glutamyl-cysteïne synthetase een betere parameter van de detoxificatiecapaciteit van de tumor. Het lijkt interessant om de expressie van deze enzymen in primaire ovariumcarcinomen te relateren aan reaktie op cytostatica. Tegelijkertijd moeten ook de resultaten afgewacht worden van klinische studies waarin de toxiciteit en effectiviteit wordt onderzocht van het toedienen van een combinatie van melfalan als alkylerend middel met buthionine sulfoximine, waarvan bekend is dat het de cellulaire glutathion gehaltes doet afnemen.

De studie naar LRP expressie in het ovariumcarcinoom laat een verrassend sterke relatie zien tussen LRP expressie en de reaktie op voornamelijk platinum bevattende cytostatica. Vooralsnog is onduidelijk hoe verhoogde LRP expressie verantwoordelijk kan zijn voor resistentie tegen platinum bevattende cytostatica. Indien echter in toekomstige prospectieve studies de prognostische waarde van LRP expressie in het ovariumcarcinoom wordt bevestigd, lijkt het van belang om te onderzoeken welke middelen in staat zijn om door LRP veroorzaakte resistentie te moduleren. De combinatie van platinum bevattende cytostatica met dergelijke middelen is wellicht werkzaam in anderszins platinum resistente ovariumcarcinomen.

Onze studie naar IL-6 in serum en cystevocht van patiëntes met benigne en maligne ovariumtumoren wijst op een mogelijke rol van IL-6, afkomstig uit de tumor, in het onstaan van algemene ziekteverschijnselen bij patiëntes, zoals laag hemo-

globine gehalte en een hoog aantal trombocyten. IL-6 is hoger in cystevocht van maligne tumoren dan van benigne tumoren. Onze studie verschaft echter geen duidelijkheid over de vraag of IL-6 wel of niet een auto- of paracriene groeifactor is in het ovariumcarcinoom. Uit experimentele studies blijkt, dat normaal p53 in staat is de expressie van IL-6 te onderdrukken Deze eigenschap gaat verloren wanneer een mutatie in het p53 gen optreedt (4). Momenteel wordt door ons onderzoek verricht in ovariumcarcinoom-cellijnen en biopsieën naar een mogelijke relatie tussen p53 expressie en IL-6 productie door ovariumcarcinoomcellen.

Het aantonen van verschillende genetische veranderingen, die betrokken zijn bij het ontstaan van het ovariumcarcinoom, dient te leiden tot nieuwe vormen van behandeling voor patiëntes met een ovariumcarcinoom. Onze studie en die van anderen laten zien dat positieve aankleuring van p53, wijzende op de aanwezigheid van een mutatie in het p53 tumor-suppressor-gen, geassocieerd is met parameters van agressieve tumorgroei en tevens met kortere overleving. Het doel van gentherapie in het geval van verlies van tumor-suppressor-genen door mutatie, is de reïntroductie van het verloren gegane tumor-suppressor-gen (5). Uit experimentele studies is gebleken, dat de transfectie van een normaal p53 gen in tumorcellen met een gemuteerd p53 gen leidt tot groeivertraging (6). Het vooralsnog meest op de voorgrond staande probleem in de toepassing van dergelijke gentherapie in de kliniek is het bereiken van alle tumorcellen. Het feit, dat de metastasering van het ovariumcarcinoom meestal beperkt is tot de peritoneumholte, kan een voordeel betekenen wanneer dergelijke therapie overwogen wordt in een in eerste instantie experimentele behandeling van patiëntes met een gemetastaseerd ovariumcarcinoom.

In experimentele studies blijkt het verlagen van overexpressie van oncogenen zoals c-erbB-2 door middel van "antigenen" eveneens een groeiremmend effect te hebben (7). De ook in onze studie gevonden frequentie van c-erbB-2 overexpressie van 20-30% in het ovariumcarcinoom, toont aan dat ook dit oncogen voor bepaalde patiëntes met ovariumcarcinoom een specifiek doel voor gentherapie kan zijn. Ook de combinatie van cytostatica met specifiek tegen groeifactor-receptoren (c-erbB-2, EGF-R) gerichte monoclonale antilichamen biedt nieuwe mogelijkheden voor behandeling.

Na de introductie van platinum bevattende cytostatica in de behandeling van patiëntes met ovariumcarcinoom zijn er de afgelopen twee decennia geen essentiële veranderingen geweest in de behandeling met cytostatica van patiëntes met een gemetastaseerd ovariumcarcinoom. De recente inclusie van taxol, en eventueel taxotère in de eerstelijns en tweedelijns behandeling van patiëntes met een ovariumcarcinoom kan de prognose van deze patiëntes misschien verbeteren. Echter, vooral de exponentiële ontwikkeling van inzichten in de cellulaire mechanismen verantwoordelijk voor ontregelde tumorgroei en resistentie tegen cytostatica, zal wellicht resulteren in een

nieuw arsenaal van geneesmiddelen met specifieke antitumoractiviteit. In de komende twee decennia kunnen wellicht meer essentiële veranderingen in de behandeling van het ovariumcarcinoom tegemoet gezien worden.

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List of abbrevations

ALDH	aldehydehydrogenase
m-AMSA	4'-(9-acridinylamino)-methanesulfon-m-aniside
ATase	O^6-alkylguanine-DNA-alkyltransferase
CDDP	cisplatin
CDNB	1-chloro-2,4,dinitrobenzene
CI	confidence interval
Cpt	camptothecin
CRP	C-reactive protein
Cy	cyclophosphamide
Dox	doxorubicin
EGF-R	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
Epi	epirubicin
FIGO	International Federation of Obstetrics and Gynecology
GCS	gamma-glutamylcysteine synthetase
GGT	gamma-glutamyl transpeptidase
GST	glutathione S-transferase
HPLC	high pressure liquid chromatography
IHC	immunohistochemistry
IL	interleukin
kDNA	kinetoplast DNA
LBA	ligand binding assay
LOH	loss of heterozygosity
LRP	lung resistance protein
MDR	multidrug resistance
MRP	MDR-associated protein
MT	metallothioneins
pBR322 DNA	plasmid BR322 DNA
PFS	progression free survival
P-gp	P-glycoprotein
PMSF	phenylmethanesulfonylfluoride
Pt	platinum
RB gene	retinoblastoma gene
RFLP	restriction fragment length polymorphisms
RR	relative risk
SDS	sodium dodecyl sulphate
TGF	transforming growth factor
Topo	topoisomerase
TVI	tumor volume index
VM-26	teniposide
VP-16	etoposide